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GENERATION OF SINGLE CHAIN FV PROTEIN A FUSION MOLECULES FOR MELANOMA THERAPY

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ABSTRACT

The high incidence rate and absence of a suitable treatment pose a great threat for melanoma patients. Surgery, the main form of treatment for clinically detectable melanoma is unsuitable for micrometastatic disease. Therefore there is a clear need for the development of new adjuvant therapies.

We have cloned RAFT3 a single chain Fv specific for melanoma associated chondroitin sulphate proteoglycan (mCSP) fused with 2 artificial *S. aureus* protein A domains (ZZ). The fusion molecule (R3ZZ) has the potential to redirect both a humoral and a cellular immune response to melanoma. R3ZZ was expressed, secreted in *E. coli* and the purified protein tested *in vitro* and *in vivo*. The binding ability of the fusion protein to bind to human IgG sepharose, mCSP and the C1q component of the complement system were determined. *In vitro* antibody dependent cell mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) assays showed that RAFT3ZZ caused 80% specific ADCC after 4 hours and 65% specific CDC after 48 hours. The ability of the fusion molecule to prevent tumour formation was tested *in vivo* in Balb/c nude mice bearing human melanoma xenografts. The dosing regimes for the therapy were also investigated but remain inconclusive. Melanoma growth retardation was observed in some experiments but not in others.

Immunocompetent CBA mice were used to evaluate the potential side effects of repeated administration of RAFT3ZZ. Tail bleeds analysed showed only a weak immune response against scFv part of the therapeutic molecule after 5 boosters. The blood samples from immunised CBA mice showed RAFT3 scFv to be much more immunogenic than the fusion molecule. This phenomenon was contrary to all published literature and thus investigated further. The therapeutic potential of RAFT3 scFv was tested *in vivo* in Balb/c nude mice with results similar to RAFT3ZZ. Extensive work was carried out to locate the epitope inducing the immune response against single chain Fv fragments. We have tentatively identified two potential epitopes responsible for the immunogenic properties of scFvs. The human c-myc detection tail and the former interphase of the V domains with the C_L and C_H1 domains.

‘Here’s your rupee...’

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Chapter 1

Introduction

1 INTRODUCTION

1.1 CANCER

The understanding of cancer and its causes has changed dramatically over the past two decades, largely due to the revolution in molecular biology that has changed the face of all biomedical research. Powerful experimental tools have made it possible to uncover and explore the complex molecular machinery to understand its operations and pinpoint the defects that cause cancer cells to proliferate abnormally (Weinberg, 2000).

1.1.1 Melanoma

John Hunter, a member of the Royal College of Surgeons in London described the first case of melanoma in 1787 (Davis *et al.*, 1980). He exemplified a *cancerous fungous excrescence* behind the jaw of a 37 year old man. Samson Handley investigated the first logical approach to surgical therapy in 1905 (Handley *et al.*, 1907). Samson Handley, a Research Fellow at Middlesex Hospital, London, spent 2 years researching the metastatic dissemination of breast cancer. In 1905 he focused his attention on a case of lymphatic spread of a secondary melanoma deposit on a woman's leg. On the basis of this case study he suggested the removal of 2 inches of subcutaneous tissue to the muscle fascia with a radical removal of lymph nodes. He published his findings in *The Lancet* in 1907. This study subsequently set the convention for the surgical management of malignant melanoma for 50 years (Eedy, 2003).

1.1.2 Epidemiology of Melanoma

Melanoma is an emerging health care problem in the USA (Devesa *et al.*, 1995). Australia, Austria, Canada, Germany, Italy, the USA and Scotland have all experienced a significant increase in the incidence rates over the past decades (Tucker *et al.*, 2003). The lifetime risk of developing melanoma is 1 in 72 (Jemal *et al.*, 2002). This is equivalent to one person dying every hour from the cancer. In the USA and other countries, the incidence of melanoma has risen more rapidly than the mortality (Jemal *et al.*, 2000; Bulliard *et al.*, 2000; Mackie *et al.*, 2002; Marks, 2002).

There have been reports of incidence rates rising more rapidly amongst men compared to women in the USA and Australia (Jemal *et al.*, 2001; Marrett *et al.*, 2001). Furthermore, a number of studies have shown that the incidence rates are the highest among older men and are rising steeply in the USA, Australia and Sweden (Jemal *et al.*, 2001; Marrett *et al.*, 2001; Mansson-Brahme *et al.*, 2002). The first encouraging report demonstrating a stabilised and decreased incidence and mortality for melanoma was reported by investigators in 1999 for the years 1990-1994 in young/middle aged patients (Hall *et al.*, 1999). However, despite this encouraging report over 7000 patients died from melanoma in the United States in the year 2000 (Zalaudek *et al.*, 2003). However, in Australia there has been some evidence of a stabilisation to decrease in rates (Marrett *et al.*, 2001). In the USA, for all ages combined, incidence rates increased significantly between 1988 and 1997 for all thicknesses of melanoma in both genders, except for thick (≥ 4 mm) melanomas amongst females (Jemal *et al.*, 2001).

1.1.3 Risk Factors

Epidemiological studies over the past several decades have identified several risk factors for developing melanoma. Melanoma is a tumour derived from activated or genetically altered epidermal melanocytes. It is the result of complex interactions between genetic, constitutional and environmental factors (Sauter *et al.*, 1998; Halpern *et al.*, 1999, Slominski *et al.*, 1998). Constitutional factors include a predisposition to burn rather than to tan in the sun (skin type), freckling, childhood and adult sunburn history and ethnic background (Gallagher *et al.*, 1995). The constitutional factor most strongly associated with melanoma risk is sun sensitivity (Elwood *et al.*, 1994). Melanoma can also arise as a result of malignant transformation of other epithelial tissues such as mucosa that synthesise the pigment melanin. The diagnosis of melanoma is based on the morphological features of a given pigmented lesion.

1.1.3.1 Sunburn

The main risk factors for melanoma are believed to be intense intermittent sun and ultraviolet radiation exposure (Elwood *et al.*, 1997, Armstrong *et al.*, 2001). Sunburns at any time of life conferred almost twofold increased risks (Elwood *et al.*,

1997). Migration studies have hypothesised that childhood may be a particularly susceptible time for sun exposure (Armstrong *et al.*, 2001; Whiteman, *et al.*, 2001). However, Elwood and co-workers (1997) found no apparent difference in risk of melanoma among individuals who sunburned in childhood, adolescence or as adults (Elwood *et al.*, 1997). These findings were confirmed in a later case control study showing no difference in risk between sunburns in childhood (less than 15 years) and older ages (Pfahlberg *et al.*, 2001). Whiteman *et al.*, (2001) concluded that even though exposure to high levels of sunlight in childhood is an important risk factor for melanoma sun exposure in adulthood is equally important. A history of sunburns, even after the age of 20, is associated with an increased risk of melanoma (American Academy of Dermatology, Press Release 2003).

Behavioural patterns may be responsible for the variation in results of different studies. A majority of an individual's total life exposure to sun is obtained during childhood and adolescent years as a larger portion of time is spent outdoors in youth compared to latter years in life (Fears *et al.*, 2002). However, regardless of whether childhood burns or adult exposure is the more important factor resulting in the onset of melanoma, it is clear that the exposure to sun and ultraviolet radiation is an important factor in the cause of melanoma.

1.1.3.2 Family History

The definition of family history of melanoma varies in different studies and geographic locations. It is a complex variable, because multiple members in a family with melanoma could represent either genetic susceptibility or common exposures to risk factors or both (Tucker *et al.*, 2003). However, family members with genetic melanoma tend to have onset earlier, thinner melanomas are detected and are more likely to develop multiple primary melanomas (Kopf, *et al.*, 1986; Barnhill *et al.*, 1993).

1.1.3.3 Skin Type and Pigmentation

Individuals that have a higher propensity to burn and freckle have a considerably higher risk of developing melanoma than those with darker skins (Bliss *et al.*, 1995; Armstrong *et al.*, 2001). People with a white racial background have a 10-fold higher

incidence of melanoma compared with blacks and a 7-fold higher incidence compared with American Hispanics residing in the same area (Horm *et al.*, 1984). Blue eyes and red or blonde hair colour, although useful as indicators of increased melanoma risk, are not as directly related to increased risk as skin type (Garbe *et al.*, 1994).

1.1.3.4 Number and Type of Nevi

A majority of all epidemiological studies carried out examining nevi have identified nevi as a risk factor for melanoma. A number of studies have reported great heterogeneity in the methods of counting nevi. These range from self-assessment to interviewer counting nevi on the arms to full body examinations by trained clinicians (Osterlind *et al.*, 1988; Aitken *et al.*, 1994; White *et al.*, 1994; Tucker *et al.*, 1997). Despite the variations in counting nevi there has been consistency in showing increased number of nevi correlates with increased risk (Bliss *et al.*, 1995). Fewer studies have assessed types of nevi (common versus atypical or dysplastic) and fewer still have been successful in determining the effects of different types of nevi (Tucker *et al.*, 2003). Where that has been possible, increased numbers of small and large banal nevi conferred moderately higher (2-4 fold increase) risks (Tucker *et al.*, 1997). These risks are on par with sun-related risk factors. Dysplastic or atypical nevi were first described in American melanoma-prone families (Clark *et al.*, 1978; Lynch *et al.*, 1978). They also occur frequently in melanoma-prone families from Scotland (MacKie, 1982), the Netherlands (Bergman *et al.*, 1992), England (Newton-Bishop *et al.*, 1994), Australia (Ang *et al.*, 1998), Sweden (Hashemi *et al.*, 1999), Italy (Landi *et al.*, 1999), Spain (Ruiz *et al.*, 1999) and France (Chaudru *et al.*, 2004). It was initially hypothesised that dysplastic nevi and melanoma were pleiotropic effects of a single gene (Bale *et al.*, 1986). However, at present the majority of data suggest that dysplastic nevi are independent risk factors for melanoma. Other risk factors include immunosuppression (e.g. renal transplant patients), previous melanoma and occupational hazards. For example, an Iceland air pilot study showed that pilots are at a more than 10-fold higher risk of developing melanoma (Pukkala *et al.*, 2002).

1.1.4 Diagnosis of Melanoma

The misdiagnosis of melanoma is a major cause of malpractice involving pathologists and dermatologists. A detailed analysis of individual surgical pathology and cytology

claims (excluding Pap smears) reported to The Doctors Company from 1995 through to 2001 revealed that 46 of 362 claims (13%) involved the misdiagnosis of melanoma and 70% of these claims were for false-negative diagnoses. Melanoma claims were second only to claims involving breast biopsy (Troxel *et al.*, 2003).

The recognition of melanoma subtypes has been linked to the concept of a biphasic natural history of melanoma: LMM (Lentigo Maligna Melanoma), SSM (Superficial Spreading Melanoma) and ALM (Acral Lentiginous Melanoma) initially extend along the epidermal surface (horizontal). A melanoma in the horizontal growth phase corresponds histopathologically to either an entirely intraepidermal (in situ) or a micro-invasive lesion. If left untreated, melanoma progresses to a deeply seated growth, which is associated with the capability to metastasise ('vertical' growth phase). Unlike the aforementioned types of melanoma, nodular melanoma has no discernible phase of horizontal growth; therefore it is rapidly progressive (Zalaudek *et al.*, 2003).

1.1.5 Melanoma Staging

The different stages of melanoma and their description are shown in Tables 1.1. and 1.2. The difference in T (tumour) stages in relation to the tumour depth, ulceration, stages, nodal mass and metastasis are based on the new American Joint Committee on Cancer staging system (AJCC) (Balch *et al.*, 2002).

Stage	Description
T stages	
Tx	Indeterminate or unknown primary
Tis	<i>In situ</i>
T1	≤ 1.0 mm depth
T2	1.01-2.0 mm depth
T3	2.01-4.0 mm depth
T4	> 4.0 mm depth
T ulceration	
Ta	No ulceration
Tb	Ulceration present: absence of epidermis over major portion of melanoma by microscopy
N stages	
N1	1 metastatic node
N2	2-3 nodes, in-transit metastases or satellite(s) without nodal metastasis
N3	4 or more nodes, matted nodes, satellite(s) or in-transit metastases with metastatic node(s)
Nodal mass	
Na	Microscopic or clinically occult
Nb	Macroscopic or clinical or radiological with pathological confirmation
Nc	Satellite(s) or in-transit metastases
Metastasis	
M1a	Skin, soft tissues, distant lymph nodes
M1b	Lung
M1c	Other visceral organs or any distant metastasis and confirmed lactate dehydrogenase elevation

Table 1.1: The new American Joint Committee on Cancer Tumour Node Metastasis melanoma staging system.

The different AJCC stages in relation to the diagnostic results as defined by the American Joint Committee on Cancer staging system for melanoma are shown in Table 1.2.

	Clinical Staging ^a			Pathologic Staging ^b		
Stage 0						
0	Tis	N0	M0	Tis	N0	M0
Stage 1 – Thin Primary						
IA	T1a	N0	M0	T1a	N0	M0
IB	T1b	N0	M0	T1b	N0	M0
	T2a	N0	M0	T2a	N0	M0
Stage II – Thick Primary						
IIA	T2b	N0	M0	T2b	N0	M0
	T3a	N0	M0	T3a	N0	M0
IIB	T3b	N0	M0	T3b	N0	M0
	T4a	N0	M0	T4a	N0	M0
IIC	T4b	N0	M0	T4b	N0	M0
III ^c	Any T	N1	M0			
		N2				
		N3				
Stage III – Spread to local lymph nodes						
IIIA				T1-4a	N1a	M0
				T1-4a	N2a	M0
IIIB				T1-4b	N1a	M0
				T1-4b	N2a	M0
				T1-4a	N1b	M0
				T1-4a	N2b	M0
				T1-4a/b	N2c	M0
IIIC				T1-4b	N1b	M0
				T1-4b	N2b	M0
				Any T	N3	M0
Stage IV – Distant spread beyond lymph nodes						
IV	Any T	Any N	Any M1	Any T	Any N	Any M1

Table 1.2: Clinical and pathological melanoma staging. ^a Clinical staging includes microstaging of the primary melanoma and clinical/radiologic evaluation for metastases. By convention, it should be used after complete excision of the primary melanoma with clinical assessment for regional and distant metastases. ^b Pathologic staging includes microstaging of the primary melanoma and pathologic information about the regional lymph nodes after partial or complete lymphadenectomy, except for pathologic stage 0 or stage IA patients, who do not need pathologic evaluation of their lymph nodes. ^c There are no stage III subgroups for clinical staging. Adapted from the American Joint Committee on Cancer Staging System for Cutaneous Melanoma (Kim et al., 2002).

1.1.6 Melanoma Staging and Survival

If detected at stage I and the depth of invasion is 0.76mm, melanoma patients have a 95% 5 year survival rate. However, the survival rate decreases to 84%, if the depth of invasion is 0.76-1.5mm. Stages II and III show typical 5 year survival rates between 60 and 70%. If the melanoma is diagnosed at stage IV, the 5 year survival is reduced to 16% (Slingluff *et al.*, 1992). If melanoma is not detected at an early stage there is a high risk of recurrence in patients. In patients with metastatic disease, chemotherapy, biologic therapy (e.g. interleukin 2 [IL-2], tumour infiltrating lymphocytes, lymphokine activated cells) and combination biochemotherapy have yielded low response rates of approximately 20-30% (Rosenberg *et al.*, 1994; Rosenberg *et al.*, 1997).

1.1.7 Different Types of Melanoma

Different types of melanoma can be identified according to their physical appearance and specific characteristics. Visual examination remains the most reliable means of identification. The ABCD rule is a clinically helpful guideline in the diagnosis of pigmented skin lesions. It is: Asymmetry, Border irregularity, Colour variation and Diameter of more than 5 mm (Friedman *et al.*, 1985). Changes in size, colour, shape of a given lesion and onset of symptoms such as itching, foreign-body sensation or bleeding are tools used for the diagnosis of amelanotic melanoma. Unfortunately, by only following the clinical ABCD rule, 1-25% of melanomas will be missed and probably a large number of benign melanocytic nevi will be unnecessarily excised.

1.1.7.1 Lentigo Maligna Melanoma

Lentigo Maligna Melanoma (LMM) accounts for approximately 5% of melanomas (Koh *et al.*, 1984). It is caused as a result of sun damage to the skin at the head and neck and arises in the elderly from lentigo malign or Hutchinson's freckle (Rippey, 1977). It appears as an irregular, pigmented and shaped macule (i.e. a flat lesion) that enlarges progressively over several years. A typical LMM is shown in Figure 1.1a. LMM is an *in situ* melanoma. Usually the horizontal growth phase of LMM is particularly prolonged. Therefore, a large number of cases are excised in a growth phase in which the prognosis is excellent (Ackerman *et al.*, 1987). However, once it

enters the vertical growth and a full-blown LMM is identified by histopathology, its prognosis becomes similar to the other subtypes of melanoma (Gaspar *et al.*, 1997; Koh *et al.*, 1984). Clinical differential diagnosis of LMM includes solar lentigo, seborrheic keratosis, pigmented actinic keratosis and basal cell carcinoma (Zalaudek *et al.*, 2003).

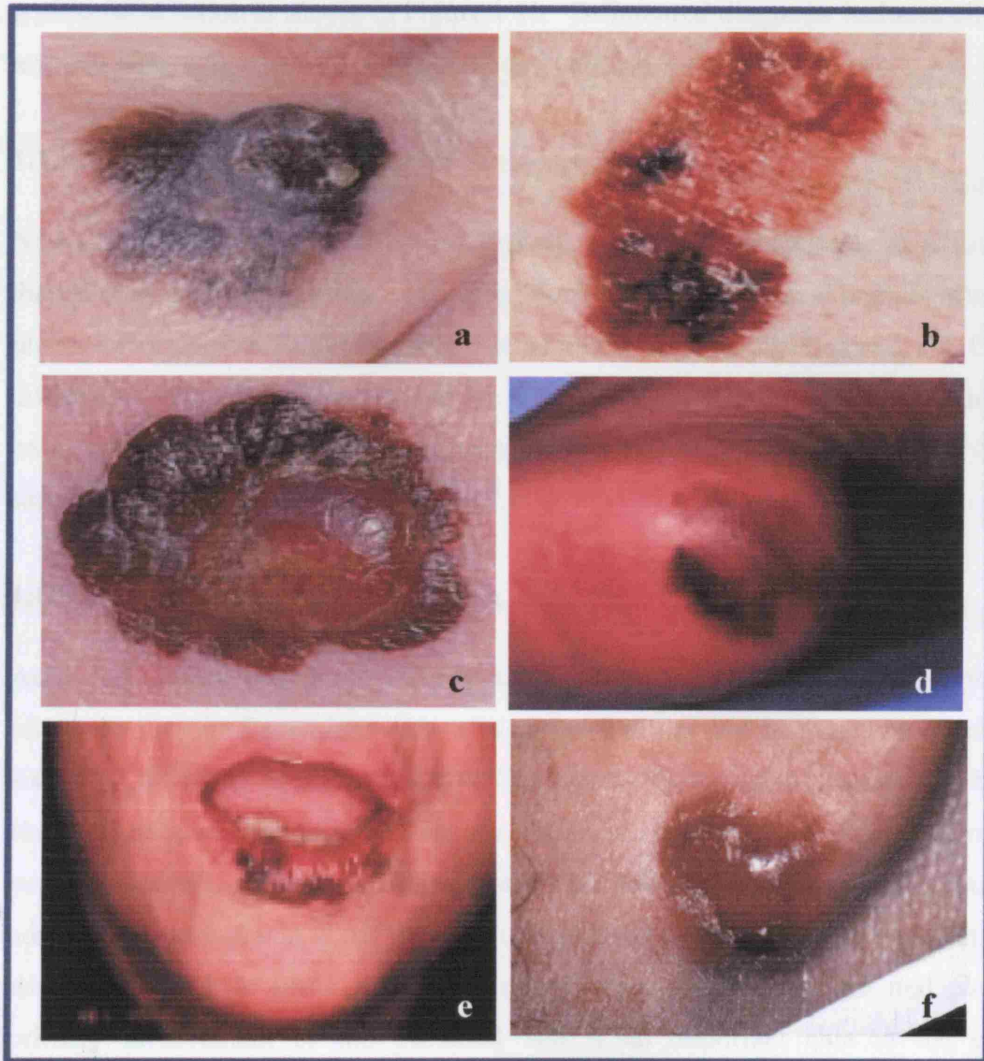


Figure 1.1: Different subtypes of melanoma (a) Lentigo Maligna Melanoma, (b) Superficial Spreading Melanoma, (c) Nodular Melanoma, (d) Acral Lentiginous Melanoma [http://tiscali.medicdirect.co.uk/self_exam/default.asp?step=4&pid=872], (e) Mucosal Melanoma [webs.comb.es/dermacat/ubderm/cirur.htm] and (f) Amelanotic Melanoma [<http://www.bccancer.bc.ca/HPI/SkinCancerAtlas/Melanoma/AmelanoticMelanoma/03.htm>].

1.1.7.2 Superficial Spreading Melanoma

Superficial spreading melanoma (SSM) accounts for approximately 70% of melanomas (Zalaudek *et al.*, 2003). It is found mainly on the trunk in men and on the leg in women (MacKie *et al.*, 1992). It appears as an irregularly shaped macule or plaque with a large variability of colours (from white to red, to brown and black). A typical SSM lesion is shown in Figure 1.1b. Differential diagnosis includes clinically atypical nevi, seborrheic keratosis and basal cell carcinoma.

1.1.7.3 Nodular Melanoma

Nodular melanoma represents approximately 15% of all melanomas. It is found on the trunk as a rapidly growing uniformly blue-black, dome-shaped nodule, often with ulceration. A typical form of nodular melanoma is illustrated in Figure 1.1c. Clinical differential diagnosis includes dermal nevi, Spitz-Reed nevi, (cellular) blue nevi, basal cell carcinoma, cutaneous adnexal neoplasms, angiokeratoma, nodular Kaposi sarcoma and other vascular neoplasms.

1.1.7.4 Acral Lentiginous Melanoma

Acral lentiginous melanoma (ALM) a unique subtype of cutaneous melanoma is relatively rare in Caucasians (2%-8% of melanomas) but is found in Blacks and Asians. It develops on the palms, soles or beneath the nail and presents as a brown-black, ill-defined macule or flat papule, which needs to be differentiated from acral nevi, viral warts, subcorneal haemorrhage (the so-called black heel) and cutaneous adnexal tumours (Saida *et al.*, 1995). Subungual melanoma typically manifests itself with a brownish to black discoloration beginning on the proximal nail plate. Its primary involvement of the proximal nail is an important clue in the clinical differential diagnosis with subungual hemorrhagia or haematoma (Shelton *et al.*, 2001). It is identified by the pigment melanin in the periungual area (Hutchinson's sign) (Kim *et al.*, 2003). Subungual melanoma is often misdiagnosed as longitudinal melanonychia, onychomycosis or nail bed nevi (Zalaudek *et al.*, 2003).

1.1.7.5 Mucosal Lentiginous Melanoma

Melanoma arising on mucosal surfaces is histopathologically similar to acral lentiginous melanoma. It is usually found on the oral, genital and anal mucosae. The flat, irregularly pigmented and shaped mucosal melanoma must be differentiated from mucosal blue nevus, mucocoele and several epithelial and vascular neoplasms (Figure 1.1e (Rosai, 1996)).

1.1.7.6 Amelanotic Melanoma

This subtype of melanoma lacks pigmentation and appears as a flat, red macule or papule (Figure 1.1f) with a broad spectrum of differential diagnoses (e.g. Spitz nevus, angioma and other vascular neoplasms, acrochordon, callus, dermatofibroma, dermatitis, basal cell carcinoma, Bowen's disease, squamous cell carcinoma, keratoacanthoma, pyogenic granuloma, cutaneous adnexal neoplasms). Amelanotic melanoma can be diagnosed by identifying a history of rapid growth or itching and bleeding. However, no single clinical feature determines a preoperative diagnosis of amelanotic melanoma.

1.1.8 Cancer and the Immune System

Clinical and pathologic observations provide evidence that malignant melanoma is an antigenic tumour recognised by specific lymphocytes. Immunity can develop to melanoma. Several melanoma-associated antigens have been shown to cause tumour rejection *in vitro* (Geertsens *et al.*, 1999). In particular, spontaneous regression of melanomas and response to immunologic therapies provide evidence that the immune system can induce regression of melanoma (Mukherji *et al.*, 1995).

1.1.8.1 Immune Reactions to Tumour Antigens

The concept of immunosurveillance postulates that transformed cells trigger an immune response that leads to the rejection of developing cancer lesions (Burnet *et al.*, 1970). It is well known that tumours frequently are infiltrated by T lymphocytes and natural killer (NK) cells (Tefany *et al.*, 1991). The quality and quantity of the immune response evoked is determined by the nature of the antigens expressed by the tumour. For example, carbohydrate antigens induce highly potent humoral immune

responses, while protein antigens trigger a cell-mediated response (Apostolopoulos *et al.*, 1999).

The immunogenicity of a particular tumour is dependent upon how it processes its antigens. In the absence of IFN- γ , the peptides presented to cytotoxic T lymphocytes (CTLs) by MHC class I molecules result from the degradation of intracellular proteins by the proteasome. However, in the presence of IFN- γ , the three catalytic subunits of the proteasome are replaced by their homologues to form the immunoproteasome (Van den Eynde *et al.*, 2001). This changes the cleavage pattern and the profile of antigenic peptides produced by the cell. Some peptides that are poorly processed by the standard proteasome are processed more efficiently and act as better stimulators of CD8⁺ T cells. However, the reverse case, where the processing efficiency of the immunoproteasome for a peptide is lower than the standard proteasome, is also known (Morel *et al.*, 2000). Therefore, the local concentration of IFN- γ has an effect on determining the immunogenicity of a tumour antigen.

The initial carcinogenic event also affects the immunogenic profile of a tumour. For instance, ultraviolet (UV) radiation induced tumours have been shown to be highly immunogenic (Kripke, 1974). Furthermore, the spontaneous regression of AK-5 melanoma (Khar, 1986; Khar, 1993) in syngeneic hosts is a consequence of the highly immunogenic nature of the tumour. The gene for the tumour rejection antigen in AK-5 has been cloned and a recombinant fusion protein found to impart immunity to animals that resisted the growth of the AK-5 tumour on subsequent challenge (Muralikrishna *et al.*, 1998). The fact that other tumours grew in the immunised animals suggests an antigen specific immunity.

In the classical pathway, soluble antigens are acquired and processed by antigen presenting cells and presented by MHC class II molecules to CD4⁺ T_H cells. The resulting activated T cells provide help to antigen-specific CD8⁺ T cells in the form of secreted cytokines and interaction of co-stimulatory cell surface molecules (Pardoll *et al.*, 1998). These CD8⁺ T cells are also directly activated by target cells presenting endogenously synthesised antigenic peptides by MHC class I molecules. This pathway functions effectively if the antigen is a soluble molecule or is released from the degraded and/or dying tumour cell. As tumour growth progresses, parts of the

tumour can become necrotic and soluble tumour antigens are released. By this time, however, the tumour could have reached a threshold size against which any immune response is impotent. Thus, for an effective anti-tumour immune response, it is necessary that the tumour antigens are accessible to the effector cells of the host at an early stage in tumour development (Mitra *et al.*, 2003).

It is now generally accepted that T cells mediate specific immune responses directed against tumour antigens. It has been possible to obtain highly specific CTLs from syngeneic animals and to use these to define tumour antigens (Kawakami *et al.*, 1994). However, MHC antigens on tumour cells are required for T-cell mediated immune defence against cancer and down-regulation of MHC expression can lead to tumour escape.

Many tumours have developed selective mechanisms for down-regulating classical MHC class I expression (Bodmer *et al.*, 1993; Garrido *et al.*, 1997) allowing them to escape CTL immunosurveillance. Such MHC class I-negative tumours consequently should become targets for NK lysis, leading to tumour regression (Porgador *et al.*, 1997). However, most tumours maintain their ability to grow *in vivo*. Furthermore, a study by Ikeda and co-workers has demonstrated that classical HLA-Cw7 class I molecules expressed on melanoma cells are able to mediate tumour escape from immunosurveillance by interacting with killing inhibitory receptors (KIRs) present on CTL (Ikeda *et al.*, 1997).

The ability of NK cells to interact with specific classical HLA class I alleles led to the identification of KIRs (Lanier *et al.*, 1997; Reyburn *et al.*, 1997; Moretta *et al.*, 1997). Numerous reports have demonstrated that the nonclassical HLA-G class I molecule inhibits NK lytic activity upon interaction with KIRs that belong to both the C-type lectin and Ig superfamilies (Pazmany *et al.*, 1996; Perez-Villar *et al.*, 1997, Munz *et al.*, 1997; Pende *et al.*, 1997; Soderstrom *et al.*, 1997). There has also been speculation regarding the existence of a different, still uncharacterized KIR able to interact specifically with HLA-G (Pende *et al.*, 1997; Rouas-Freiss *et al.*, 1997). Thus, expression of HLA-G by target cells would represent a compelling mechanism that tumours utilise to escape from NK cell immunosurveillance (Paul *et al.*, 1998).

In contrast to classical HLA class I genes, the primary transcript of the HLA-G gene generates at least five different mRNAs resulting from alternative splicing that potentially encode five protein isoforms: HLA-G1, HLA-G2, HLA-G3, and HLA-G4, which are anchored to the cell membrane and the soluble HLA-G5 isoform (Ishitani *et al.*, 1992, Kirszenbaum *et al.*, 1994). It has been shown that the HLA-G1 and HLA-G2 isoforms strongly inhibit NK cell lysis *in vitro* (Rouas-Freiss *et al.*, 1997a) and that HLA-G molecules protect cytotrophoblasts from the lytic activity of maternal uterine NK cells *ex vivo* (King *et al.*, 1997; Rouas-Freiss *et al.*, 1997b).

In order to characterise the ectopic expression of HLA-G on tumour cells as a means of escape from immunosurveillance Paul *et al.* (1998) studied HLA-G isoform expression and function in human melanoma cell lines. To demonstrate the biological relevance of HLA-G expression in tumours, the authors also carried out *ex vivo* experiments on melanoma metastasis biopsies. They concluded that HLA-G is expressed in solid tumours and can protect them from NK cell lysis (Paul *et al.*, 1998).

1.1.8.2 Immunosuppression

Some tumours are immunosuppressive rather than immunogenic. These tumours produce cytokines, growth factors, chemotactic molecules and proteases that inhibit cell mediated immunity (Ting *et al.*, 1979). Many tumour-derived molecules, such as IL-4, IL-6, IL-10, TGF- β 1, prostaglandin E2 (PGE2) and macrophage colony stimulating factor (M-CSF) deactivate or suppress immune cell cytotoxicity (Elgert *et al.*, 1998). Other tumour cells avoid confrontation with host effector cells by secreting anti-inflammatory factors that inhibit host cell migration across the vascular endothelium into the tumour mass (Fauve *et al.*, 1974).

In addition to the direct secretion of immunosuppressive cytokines, cancer cells can induce host cells to release immune inhibitors (Huang *et al.*, 1998). Tumour-derived prostaglandins augment the production of inhibitory cytokines such as IL-10 (Huang *et al.*, 1996), while suppressing endogenous production of cytokines including IL-12 that are necessary for effective host-cell-mediated antitumour immune responses (Van Der Pouw Kraan *et al.*, 1995). The enzyme cyclooxygenase 2 (COX-2) acts at the rate

limiting step of prostanoid production (Smith, *et al.*, 1996). Therefore, COX-2 expression by tumour cells might be an important therapeutic target for pharmacological or gene therapy intervention.

TGF- β is one of the most potent immunosuppressive factors known (Wahl *et al.*, 1989). It affects proliferation, activation and differentiation of the cells that participate in both innate and acquired immunity. TGF- β inhibits immunoregulatory cytokine production (Espevik *et al.*, 1987) including IL-12 production by monocytes (Ruegemer *et al.*, 1990). TGF- β has also been reported as a potent inhibitor of CTL differentiation (Chang *et al.*, 1993) suggesting that this factor might affect the development of tumour-reactive CTLs *in vivo*. However, since TGF- β is also a potent inhibitor of the proliferation of neoplastic cells *in vitro* (Moses *et al.*, 1987), this cytokine might exert dual effects on tumour growth *in vivo*.

Tumour associated macrophages (TAMs) that are capable of suppressing lymphocyte responses have been known for some time (McBride *et al.*, 1986). A study by Evans and co-workers (1984) showed that TAMs were able to enhance or suppress *in vitro* proliferative responses to mitogens depending on the stage of tumour growth. Macrophages from early or late tumours were immunosuppressive, whilst those in mid-stage tumours enhanced immune responses. Such changes in TAM function seemingly reflect the self-regulatory potential of the macrophage system (Gillespie *et al.*, 1980). Macrophages release reactive nitrogen and oxygen intermediates (RNI and ROI), which directly suppress other immune cells, and TNF- α , which suppresses indirectly by inducing production of inhibitory molecules such as PGE2 by macrophages (Taffet *et al.*, 1981). Although the cytotoxic activity of RNI, ROI and TNF- α might benefit the tumour-bearing host, the suppressor activity of these molecules may prevent antitumour lymphocyte responses. However, there is no evidence suggesting the existence of suppressor macrophages that are able to exert their influence on immunosuppressive mechanisms to initiate immune evasion (Bhaumik *et al.*, 2001).

1.1.9 Melanoma During Pregnancy

Allen and co-workers published a report suggesting that there could be spontaneous regression of melanoma following pregnancy (Allen *et al.*, 1955). However, a majority of the published literature contradicts this finding. Since 1951, a number of case reports have suggested that pregnancy may have an adverse effect on the clinical course of malignant melanoma. Slingluff and co-workers studied patients diagnosed with melanoma during pregnancy and followed up for a mean of 6.8 years. The authors found that compared with non-pregnant female patient population there was a significantly shorter disease-free interval for the pregnant group. The median disease-free intervals observed were 5.8 and 11.9 years, respectively (Slingluff *et al.*, 1992). Grin *et al.*, (1996) reviewed the literature on pregnancy and melanoma and concluded that based on a limited number of controlled trials, pregnancy before, after or during the time of diagnosis of stage I malignant melanoma does not appear to affect survival (Grin *et al.*, 1996). A more recent study by Daryanani (2003) suggested that women who present with melanoma during pregnancy have a worse prognosis due to more aggressive behaviour of the melanoma. However, the authors concluded that pregnancy does not appear to have a long-term adverse effect on survival in patients with clinically localised melanoma.

1.1.10 Tumour Associated Antigens

During the last decade significant progress has been made with the identification of melanoma-associated antigens (MAA) recognised by cytotoxic T lymphocytes (CTLs). These antigens belong to three main groups: tumour-associated cancer testis-specific antigens (e.g. MAGE, BAGE, GAGE and PRAME), melanocyte differentiation antigens (e.g. tyrosinase, Melan-A/MART-1, gp100, TRP-1 and TRP-2) and mutated or aberrantly expressed self antigens (e.g. MUM-1, CDK4, β -catenin, gp100-in4, p15 and N-acetylglycosaminyltransferase). A large number of T cell epitopes have been identified for these antigens, opening up new possibilities for the immunotherapy of malignant melanoma (Kirkin *et al.*, 1998). Table 1.3 illustrates these groups of antigens.

Antigen	HLA	Epitope	Sequence
<i>Testis-specific antigens</i>			
MAGE-1	A1	161-169	EADPTGHSY
	Cw16	230-238	SAYGEPKRL
MAGE-3	A1	168-176	EVDPIGHLY
	A2	271-279	FLWGPRLV
	B4	167-176	MEVDPIGHLV
	A2	195-203	IMPKAGLLI
MAGE-6	Cw1601	293-301	ISGGPRISY
BAGE	Cw16	2-10	AARAVFLAL
GAGE-1	Cw6	9-16	YRPRPRRY
PRAME	A24	301-309	LYVDSLFFL
<i>Melanocyte differentiation antigens</i>			
gp100	A2	154-162	KTWGQYWQV
	A2	209-217	ITDQVPFSV
	A2	280-288	YLEPGPVT A
	A2	457-466	LLDGTATLRL
	A2	476-485	VLRYGFSFSV
	A3	17-25	ALLAVGATK
	A11	87-95	ALNFPGSQK
	A24	intron 4	VYFFLPDHL
	A68	182-191	HTMEVTVYHR
	Cw8	71-78	SNDGPTLI
MART-1	A2	27-35	AAGIGILTV
	A2	32-40	ILTVILGVL
	B45	24-33(34)	AEEAAGIGIL(T)
	D	51-73	RNGYRALMDKSLHVGTCALTRR
Tyrosinase	A2	1-9	MLLAVLYCL
	A2	369-377	YMNGTMSQV
			YMDGTMSQV
	A24	206-214	AFLPWHLRF
	B44	192-200	SEIWRDIDF
TRP-1/gp75	A31	1-9	MSLQRQFLR
TRP-2	A31	197-205	LLPGGRPYR
<i>Mutated and aberrantly expressed antigens</i>			
MUM-1	B44	782-808	EEKLIVVLF
b-Catenin	A24	29-37	SYLDSGIHF
P15	A24		AYGLDFYIL
CDK4	A2		ACDPHSGHFV
GnT-V	A2		VLPDVFIRC
gp100-in4	A24	170-178	VYFFLPDHL

Table 1.3: Antigenic epitopes of melanoma associated antigens recognised by CTL (adapted from Zeuthen *et al.*, 1998; Kawashima *et al.*, 1998; Robbins *et al.*, 1997; Sensi *et al.*, 2002; Castelli *et al.*, 1999; Schneider *et al.*, 1998; Zarour *et al.*, 2000; Vantomme *et al.*, 2003).

1.1.10.1 Tumour Associated –Cancer Testis Specific Antigens

Tumour associated cancer specific antigens are proteins expressed in many tumours but only in few normal tissues (testes and in some cases in the placenta). This group of antigens includes the MAGE, BAGE, GAGE and PRAME antigens. The MAGE genes represent a family of 12 closely related genes located on the long arm of chromosome X (DePlaen *et al.*, 1994). The MAGE proteins are relatively large proteins with a number of potential HLA class I binding epitopes. However, peptide

epitopes recognised by CTLs have been identified only for MAGE-1 (Van Der Bruggen *et al.*, 1994) and MAGE-3 (Gaugler *et al.*, 1994) and MAGE-6 (Table 1.3).

The expression of MAGE antigens has been further investigated in a number of studies. These antigens are generally highly expressed in cutaneous melanomas (up to 65 % for MAGE- 3) but not in ocular melanomas (DePlaen *et al.*, 1994; Mulcahy *et al.*, 1996). To a lesser extent they are expressed in other types of tumours such as mammary carcinomas, head and neck tumours, lung carcinomas, sarcomas and bladder carcinomas (Zeuthen *et al.*, 1996). The expression of MAGE-1 in melanoma is more frequent in metastases than in primary tumours (Brasseur *et al.*, 1995).

MAGE antigens represent very good targets for immunotherapy. They are widely distributed in a number of tumours but not expressed in normal tissues. MAGE antigens present only in testis a tissue which is not accessible to the cells of the immune system due to the lack of the direct contact of testis cells with immune cells (Barker *et al.*, 1977) and the lack of HLA class I expression on the surface of germ cells (Tomita *et al.*, 1993). Germ cells are the only cells in the testis expressing MAGE antigens (Takahashi *et al.*, 1995).

The testis-specific antigens such as MAGE and PRAME should potentially be highly immunogenic antigens. Despite extensive studies (van der Bruggen *et al.*, 1994, Traversari *et al.*, 1992; Gaugler *et al.*, 1994; Zorn *et al.*, 1999), however, CTL responses to MAGE antigenic peptides have been detected rarely in cancer patients, even after vaccination (Marchand *et al.*, 1995). This could possibly be due to a very low frequency of CTL precursors (Chaux *et al.*, 1998). Taken together, these observations raise doubts about the immunogenicity of MAGE antigens (Valmori *et al.*, 2001).

A comparison of the predicted secondary structures of two groups of antigens (testis-specific and melanocyte differentiation antigens) revealed an enrichment of long alpha-helical stretches in the testis-specific antigens. Kirkin and co-workers hypothesised that such highly organized structures could diminish the efficiency of protein unfolding, a necessary step in the proteolytic cleavage by proteasomes and

therefore, could be responsible for the low immunogenicity of these proteins (Kirkin *et al.*, 1998).

The expression pattern of BAGE antigens is similar to that of MAGE antigens but with a lower frequency. MAGE is expressed in melanoma (20%), in bladder carcinomas (15%), in mammary carcinomas (10%) and in head and neck squamous cell carcinomas (8%). Like MAGE antigens the expression of BAGE also correlates with the state of tumour progression (Zeuthen *et al.*, 1998).

1.1.10.2 Melanocyte Differentiation Antigens

The recognition of normal melanocyte gene products has been demonstrated for a number of CTL lines and clones and indicates that there is no strong tolerance to this group of self-proteins (Anichini *et al.*, 1993). CTLs against several melanocyte differentiation antigens have been identified so far, including tyrosinase, Melan-A/MART-1, gp100, TRP-1 and TRP-2.

Tyrosinase derived epitopes are recognised by HLA-A2.1 restricted CTLs. The generation of a tyrosinase specific cytotoxic CTL response in melanoma patients is infrequent and is usually seen only in patients showing a high response against several melanoma-associated antigens (Zeuthen *et al.*, 1998). Examples of this include patients MZ2 (Traversari *et al.*, 1992), 888 (Robbins *et al.*, 1995) and SK29 (Wolfel *et al.*, 1994) from whom several melanoma specific CTL lines have been isolated.

Melan-A/MART-1 is a small transmembrane protein that is widely distributed in melanomas but not expressed by other tumours. The relative immunogenicity of Melan-A/MART-1 is thought to be the highest among the melanocyte-specific differentiation antigens, CTL recognising peptides derived from this protein are easily induced after stimulation of PBLs with synthetic peptides or allogeneic melanoma cells (Rivoltini *et al.*, 1995; Stevens *et al.*, 1995). However, a study by Kawakami *et al.* (1995) showed that the ability of TILs (tumour infiltrating lymphocytes) to induce tumour rejection did not correlate with the recognition of Melan-A/MART-1 suggesting that the role of this antigen in the generation of protective immunity is probably not significant. Cormier *et al.* (1997) confirmed this by demonstrating that

increasing the frequency of Melan-A/MART-1-specific CTL did not induce tumour regression.

The melanocyte lineage specific antigen gp100 is recognised by HLA-A2 and HLA-A3-restricted CTLs. A correlation has been shown between T cell recognition of gp100 peptides *in vitro* and tumour regression in patients receiving TIL therapy (Kawakami *et al.*, 1995) suggesting that this antigen is a promising target for immunotherapy. However, as for tyrosinase, the same TIL cultures recognised other antigens that could induce regression, either alone or in combination with CTL clones recognising gp100. Nevertheless, this antigen is a promising HLA-A2-restricted differentiation antigen for immunotherapeutic applications.

The immunogenicity of melanocyte differentiation antigens is low (Kirkin *et al.*, 1998). This can be explained by immunological tolerance against the potential highly immunogenic epitopes of these self-proteins. The majority of peptide epitopes have low or intermediate affinity for HLA class I and the peptides can therefore only activate peptide-dependent lysis by CTL clones at relatively high concentrations (Wolfel *et al.*, 1994; Kawakami *et al.*, 1996).

T cell anergy is probably also an important factor for the low immunogenicity of melanoma differentiation antigens. It may be possible to make lymphokine activated killer cells (LAKs) reacting with gp100. Artificial gp100 peptides with better MHC anchor residues have been made that show higher affinity and mediate better kill when used for pulsing dendritic cells (Tsai *et al.*, 1997).

1.1.10.3 Mutated Self Proteins – Unique Antigens

Mutated self-antigens are usually present in primary tumours and could play an important role in the generation and/or progression of tumours. It is important to characterise these antigens not only in connection with immunotherapy but also in order to understand their involvement in the molecular mechanisms of malignant transformation. With respect to melanoma, 6 antigens belonging to this group have been characterised (Zeuthen *et al.*, 1998).

The most common antigens recognised by T cells in this group include cdk-4, β -catenin and MUM-1. The first unique antigen identified with well-known biological function was mutated cdk-4 (Wolfel *et al.*, 1995). It is a key protein involved in the regulation of cell cycle progression as a part of the cdk-4- p16-Rb pathway. It has been shown to be inactivated in human melanomas (Bartkova *et al.*, 1996).

The MUM-1 (melanoma ubiquitous mutated) antigen was identified as an HLA-B44 restricted antigen expressed by the melanoma cell line LB33-MEL (Coulie *et al.*, 1995). It is encoded by a gene that is expressed in many normal tissues. The function of this protein remains unknown. More than one third of the CTL clones established against the melanoma cell line LB33-MEL was directed against this antigen indicating the high immunogenicity of this mutated epitope (Lehmann *et al.*, 1995). The aberrant MUM-1 mRNA was observed in about 50 % of melanomas tested (Braisl *et al.*, 1998).

1.1.10.4 Cell Substrate Interacting Oncofoetal Proteins

MAA expressed by foetal cells of different embryological origin and cancer cells are known as oncofoetal antigens. In culture, only neural crest-derived cells such as melanoma and glioma express these high molecular weight antigens (Chi *et al.*, 1997). There is little heterogeneity in the expression of oncofoetal proteins between different melanomas and within each tumour. One high molecular weight antigen belonging to this class is the melanoma associated chondroitin sulphate proteoglycan (gp250) that consists of an approximately 250kDa core glycoprotein and non-covalently linked 400 kDa carbohydrate subunit. The melanoma associated chondroitin sulphate proteoglycan (mCSP) encoding gene has been mapped to chromosome 15 (Rettig *et al.*, 1986). The antigen is a major MAA that has frequently been used to generate MAbs due to its strong immunogenicity in mice (Bumol *et al.*, 1983; Herlyn *et al.*, 1987). For production of MAbs, mice were injected with melanoma cells (Koprowski *et al.*, 1978; Natali *et al.*, 1983; Bumol *et al.*, 1982; Houghton *et al.*, 1982), nevus cells or glioma cells (Cairncross *et al.*, 1982). In more than 90% of melanoma cultures, 80 to 100% of cells express mCSP (Herlyn *et al.*, 1980; 1983; 1985).

Most melanoma cells express between 100,000 and 6,000,000 mCSP molecules (Herlyn *et al.*, 1985; Giacomini *et al.*, 1985; Ziai *et al.*, 1987). Chondroitin sulphate proteoglycan is expressed on the melanoma cell surface on micro spikes which are present as 1-2 μm structures on the upper cell surface and as structures up to 20 μm at the cell periphery (Garrigues *et al.*, 1986). Expression of mCSP could be induced in human/mouse neuroblastoma cell line hybrids when cells were cultured on extra cellular matrix instead of on plastic indicating that cell-matrix interactions provide controlling signals for expression (Rettig *et al.*, 1986).

Several distinct epitopes have been detected with different MAbs on mCSP with two (Garrigues *et al.*, 1986; Ross *et al.*, 1984; Harper *et al.*, 1984), three (Ziai *et al.*, 1987; Garrigues *et al.*, 1986), and five (Rettig *et al.*, 1986) determinants defined. Antigenic determinants are located both on the 250 kDa core glycoprotein and on the > 400 kDa mCSP (Bumol *et al.*, 1982; 1984), with the epitope expression heterogeneity largely due to variable glycosylation of the molecule (Garrigues *et al.*, 1986).

1.1.10.5 Immunological Ignorance of Tumour Associated Antigens

Silent tumour antigens are naturally presented MHC class I-associated peptides that do not trigger an immune response against the tumour (Miller *et al.*, 1992). Studies using transgenic mice have shown that viral antigens expressed on pancreatic β -cells cannot delete or anergise peripheral responder CTLs, resulting in the coexistence of cells expressing these antigens with CTLs that are fully competent to reacting with the antigens *in vitro*. This phenomenon is called immunological ignorance (Miller *et al.*, 1992).

In the majority of cancer patients freshly isolated tumour infiltrating T cells are inactive against autologous cancer cells, although these cells can be activated *in vitro* with lymphokines such as IL-2 (Rosenberg *et al.*, 1998). Many factors may contribute to antigen silencing in cancer cells including the nature of the TCR ligand (i.e. peptide-MHC) and the threshold level of TCR occupation. There is a critical threshold of the number of TCR molecules that need to be engaged with peptide-MHC in order to achieve a significant and detectable T-cell response (Miller *et al.*,

1992). The threshold level depends on the type of APC and the status of the T cell (naive versus memory).

HLA-A2/A28 and HLA-B12/44/45 are potent presenting molecules for melanoma associated epitopes and may permit CTLs from patients sharing these alleles to recognise and kill melanoma cells efficiently after immunisation with an allogeneic vaccine (Kan-Mitchell *et al.*, 1993). One mechanism used by tumour cells, especially virally induced tumours, to evade the immune system is the downregulation of MHC class I expression (Ishido *et al.*, 2000). This prevents the presentation of processed tumour specific antigenic peptides by MHC molecules, which is required for the CTL response.

Another mechanism for immunological ignorance is shedding of TAAs, which prevent the tumour cell from being recognised as foreign by the immune system. This has been found for human bladder cancer (Libert *et al.*, 1989) and melanoma (Gupta *et al.*, 1984). Tumours have also been shown to escape immune destruction by masking their surface antigens with glycocalyx molecules under immunological pressure (Boyse *et al.*, 1967).

1.1.10.6 Alteration of the Tumour Microenvironment by Shed TAAs

Some TAAs are continuously shed from the tumour cell surface and bind to reticuloendothelial cells and antibodies, causing immunosuppression (Bergelson *et al.*, 1990; Portoukalian *et al.*, 1989). Gangliosides, glycosyl-phosphatidyl-inositol anchored proteins such as CEA-11 and other TAAs restricted to the outer layer of the bilayered lipid membrane are more likely to be shed and alter the tumour microenvironment. Lymphocytes recovered from tumour tissues, in contrast to those in peripheral blood, proliferate less readily in response to interleukin-2 (IL-2) or mitogens. Peripheral blood lymphocytes exposed to tumour cells or to their shed products show significantly reduced proliferation (Miescher *et al.*, 1986). However, repeated washing of the peripheral blood lymphocytes from cancer patients can restore and enhance lymphocyte killing of tumour cells (Currie *et al.*, 1973). Suppression of lymphocytes within the lymph nodes of melanoma patients correlates inversely with the distance of the nodes from the primary tumour (Cochran *et al.*,

1987). Furthermore, immunosuppression is worse in late stage disease and when the overall tumour burden is high (Gabrilovich *et al.*, 2003).

The presence of circulating TAA-antibody complexes in cancer patients who have not received immunotherapy suggests an early endogenous antibody response against the tumour (De Kernion *et al.*, 1974; Hakansson *et al.*, 1985). As the tumour grows and sheds more antigens, these antigens bind and mask free antitumour antibodies (Lewis *et al.*, 1971). It has also been shown that surgical removal of the neoplasm can reverse this immunosuppression (Morton *et al.*, 1999). Thus, successful resection of primary sarcoma was associated with a four-fold rise in serum antitumour antibody titre (De Kernion *et al.*, 1974).

1.1.10.7 Antigen Alteration and Immunologic Heterogeneity

Although it is thought that a malignant neoplasm usually evolves from a single transformed cell, most cancers are actually composed of genetically unstable populations of proliferating cells that become heterogeneous over time (Heppner *et al.*, 1984). Heterogeneity amongst tumour cells enables subsets of the population to evade the host's immune response and therefore become resistant to therapies (Nowell *et al.*, 1986). Tumours recurring at the resection site of a primary neoplasm can differ antigenically from the primary tumour. This is consistent with the possibility that antigenic variants have been selected for their ability to escape immune surveillance (Chan *et al.*, 1998). Gangliosides are an example of heterogeneous antigen expression in human melanoma. GM3 constitutes 95% of the gangliosides on melanocytes, the progenitors of human melanoma (Carubia *et al.*, 1984). During the early, radial phase of growth neoplastically transformed melanocytes express more GD3, a derivative of GM3, than normal progenitors (Ravindranath *et al.*, 1988). After the appearance of GD3, the tumour cells begin to migrate vertically. Subsequently, levels of GD3 continue to increase and other derivatives of GD3, namely, GD2 and O-AcGD3, begin to appear (Ravindranath *et al.*, 1985; 1988; 1989). These alterations in ganglioside expression correlate with and may facilitate tumour invasion and metastasis formation. During the progression of melanoma, there are similar alterations in expression of MHC antigens (HLA-DR),

intercellular adhesion molecules (ICAM-1) and mucins (MUC-18) (Stade *et al.*, 1990).

1.1.11 TAP Deficiencies

Down-regulation of MHC class I antigen expression is often associated with impaired TAP (transporters associated with antigen processing) expression in a variety of tumours (Chen *et al.*, 1996; Seliger *et al.*, 1998; 2000). There is limited information underlying the molecular mechanisms of these TAP deficiencies (Ritz *et al.*, 2001).

Most investigations have been limited to the analysis of TAP1 expression due to the limited availability of anti-TAP2 antibodies. In comparison to autologous normal tissues, TAP1 downregulation or loss of expression was found in most tumour types analysed, e.g. breast carcinoma, melanoma, cervical carcinoma and RCC. (Seliger *et al.*, 2000). Similar results were obtained by analysis of various tumour cell lines. Typical results from a study by Ritz *et al.*, (2001) are shown in Table 1.4.

The impact of TAP deficiencies by malignant cells varies amongst tumours. The frequency of TAP1 downregulation was more prominent in metastatic than primary lesions in breast, cervical, colon, small cell lung carcinomas (SCLC) and melanomas. Deficient TAP expression of renal cell carcinomas (RCC) was not correlated with tumour grading and staging (Seliger *et al.*, 1998, Kaklamanis *et al.*, 1995, 1994, Kageshita *et al.*, 1999). In melanoma, impaired TAP expression is also associated with poor prognosis and reduced survival rate (Kageshita *et al.*, 1999).

Until recently, tumour lesions/cell lines have not been monitored for TAP mutations. In one lung carcinoma cell line, a new TAP1 allele has been identified that results in expression of a nonfunctional TAP protein (Chen *et al.*, 1996). In addition, a single base pair deletion in the gene encoding the TAP1 subunit of a melanoma cell line results in TAP dysfunction accompanied by abnormalities of MHC class I surface expression (Seliger *et al.*, 2001). Seliger and co-workers (2003) showed that TAP abnormalities do not always appear to be attributable to structural alterations because no mutations in TAP1 were detected in TAP1-deficient RCC lesions. The authors

suggested that TAP defects in RCC lesions are caused by regulatory abnormalities (Seliger *et al.*, 2003).

Downregulation of Expression (%)						
Tumour Type	Primary Lesions			Cell Lines		
	Samples (n)	TAP1	TAP2	Samples (n)	TAP1	TAP2
Breast carcinoma	63	13-44	21	ND	ND	ND
SCLC	93	38	ND	6	100	100
Colon Carcinoma	81	14	ND	5	32	23
RCC	70	52	ND	19	79	74
Cervical Carcinoma	76	49	ND	7	0	0
Melanoma	53	31	16	9	33	57

Table 1.4: TAP downregulation in lesions and cell lines derived from human tumours of distinct histology. SCLC: Small cell lung carcinomas, RCC: Renal cell carcinoma, ND: not determined (adapted from Rütz, *et al.*, 2001).

Because correction of reduced TAP expression can be achieved in many tumour cells of different origin by cytokine treatment, TAP abnormalities mainly appear to be due to defects in regulatory mechanisms rather than structural alterations (Seliger *et al.*, 1996; 1997; Kaafelz *et al.*, 1999). This hypothesis is further supported by recent data from Zhu *et al.*, (1999) demonstrating that p53 as well as DNA damaging agents enhance TAP1 expression. Because more than 50% of human tumours exhibit mutations of the p53 tumour suppressor gene, it is anticipated that these p53 mutations abrogate immune surveillance. Thus, dysfunctional p53 is not capable of inducing TAP1 expression upon genotoxic stress (Zhu *et al.*, 1999). The impact of TAP abnormalities on the recognition of tumour cells by CD8⁺ CTL has only been investigated in a few cases using tumour cell lines, surrogate antigens and HLA class I restricted antigen-specific CTL (Seliger *et al.*, 1997). A correlation between the degree of TAP downregulation, impaired MHC class I surface expression and the

recognition of tumour cells by tumour antigen-specific MHC class I-restricted cells has also been reported (Seliger *et al.*, 1997; Restifo *et al.*, 1993).

A study by Qin *et al.* (2002) concluded that although the lack of TAP1 expression did not change the immunogenicity of tumour cells it could allow them to escape T cell recognition during the effector phase of an antitumour immune response.

1.1.12 Fas Ligand

The expression of Fas ligand that is involved in apoptosis has been proposed as a novel tumour escape mechanism for melanoma. Characteristics of Fas ligand expression during the course of melanoma progression as well as subsequently evolving metastases have been studied by Terheyden *et al.* (1999). The authors observed Fas ligand expression in 10% of lesions and found it was weak and restricted to few parts of the tumours. The primary melanomas that were positive for Fas ligand were fairly thick. In contrast, for metastatic melanoma Fas ligand expression was present in six of 11 cases investigated. Furthermore, apoptotic cells (less than 5%) were present within the Fas ligand positive tumours (Terheyden *et al.*, 1999). Thus, Fas ligand mediated apoptosis is not a major immune escape mechanism for melanoma but its expression correlates with the stage of melanoma. The inactivation of neutrophils by tumour FasL has also been shown to be an important mechanism by which tumour cells escape immune attack (Chen *et al.*, 2003).

1.1.13 Complement Escape

Both normal and tumour cells are protected from complement attack by different membrane-bound complement inhibitory proteins. In humans, complement activation is controlled primarily by the cell surface proteins DAF and MCP (Caragine *et al.*, 2002). Membrane-bound complement inhibitors protect host cells from inadvertent complement attack, and complement inhibitors are often up-regulated on tumours (Caragine *et al.*, 2002). Complement inhibitors have been identified as tumour-associated antigens (Kinders *et al.*, 1998; Li *et al.*, 2001) and their expression is up-regulated on some tumour cells. There is *in vitro* evidence to support the hypothesis that complement inhibitors expressed on tumour cells can provide protection from immune surveillance and that they can interfere with antibody-mediated

immunotherapy (Maio *et al.*, 1998; Gorter *et al.*, 1999; Jurianz *et al.*, 1999). Although it remains unclear why CD55 (DAF) is up-regulated in the tumour environment its high level of expression on tumour cells identifies it as a potential cause for immune escape and target for immunotherapy (Li *et al.*, 2001). The complement system and its importance in immunotherapy is discussed in detail in Chapter 3 Sections 3.1.7 - 3.1.15.

1.2 CURRENT THERAPIES

When melanomas are recognised at an early stage, i.e. in the radial growth phase and the disease is still localised and restricted to the skin, excision can result in clinical cure (Slominski *et al.*, 2001). When lesions are less than 2mm in depth (Stage I) they are predominantly curable by surgery alone. However, later stages of melanoma require adjuvant therapy following surgery. Table 1.5 illustrates the different stages of melanoma, the disease symptoms and course of treatment.

Stage	Disease	Treatment
I	Primary melanoma 2.0 mm thick without ulceration Or ≤ 1 mm thick, with ulceration, no nodal or distant metastasis	Wide local excision with 1 –2cm margins, with or without selective node mapping
II	Primary melanoma ≥ 2.0 mm thick, Or >1 mm thick, with ulceration, no nodal or distant metastasis	Wide local excision, margins 2 cm for tumours <4 mm, 2-3cm for tumours invading >4 mm, selective lymph node mapping
III	Any primary melanoma plus any regional lymph node and / or in transit metastasis	Wide local excision plus therapeutic lymph node dissection with or without interferon α
IV	Any primary tumour plus distant metastases, with or without nodal involvement, distant metastases	Chemotherapy, immunotherapy, surgery of involved sites

Table 1.5: Staging and treatment of melanoma. The 4 stages of melanoma, their disease state and most appropriate course of treatment (Adapted from Balch *et al.*, 2000).

1.2.1 Surgery

Surgical excision is the main form of treatment for melanoma. Surgical intervention for these cancers is recommended in almost all cases. In the majority of cases surgery for primary melanoma is carried out in outpatient clinics under local anaesthesia. Dermatologists are performing an increasing proportion of primary melanoma excisions. In 1979 in West Scotland dermatologists removed only 3% of all primary melanomas. By 1998 this figure had risen to 40%. Plastic surgeons now excise 26% of primary melanomas compared with 65% 20 years ago, and general surgeons excise

34% compared with 32% (Mackie *et al.*, 2002). Surgery is highly curative in the early stages, with 5-year survival rates between 92-95% for primary melanoma. The recommended excision margins from different organisations for various tumour thicknesses are illustrated in Table 1.6. The margins recommended by the Dutch MSG (melanoma study group) are narrower when compared with the Australian MSG and World Health Organisation.

Tumour thickness	<i>Recommended Excision margin</i>			
	U.K. MSG	WHO	Australian MSG	Dutch MSG
<i>In situ</i>	2-5 mm	5 mm	5 mm	2 mm
< 1 mm	1 cm	1 cm	1 cm	1 cm
1-2 mm	1-2 cm	1 cm	1 cm	1 cm
2.1-4 mm	2-3 cm (2 cm preferred)	2 cm	1 cm	2 cm
> 4 mm	2-3 cm	2 cm	2 cm	2 cm

Table 1.6. Currently recommended excision margins for primary melanoma: MSG: Melanoma Study Group; WHO: World Health Organisation (Adapted from Zalaudek *et al.*, 2003).

1.2.2 Chemotherapy

Over the past thirty years a number of approaches have been tested in an effort to reduce the risk of recurrence in high-risk populations. One such approach is chemotherapy (Karakousis *et al.*, 1987). Despite the fact that chemotherapy remains the most successful option for the treatment of many types of tumours, in melanoma patients drug therapies often fail for various reasons (Scotto *et al.*, 2000). Melanoma is a relatively chemo-resistant tumour. In addition problems inherent in melanoma chemotherapy including the lack of specificity of chemotherapeutic agents, chemo-resistance and the escape of noncycling dormant tumour cells (De Vita, 1993).

1.2.2.1 Single Agent Chemotherapy

Dacarbazine, the most active single agent in patients with metastatic melanoma, induces a partial response in less than 20% of patients and a complete response in less than 5% (Legha *et al.*, 1989). Multidrug regimens have shown somewhat higher partial response rates (25 to 40%) but have similar complete response rates of 5% (McClay *et al.*, 1992).

Other single-agent chemotherapy agents including platinum compounds, vinca alkaloids, nitrosoureas and taxanes have similar activity as dacarbazine and have not improved clinical outcome (Lotze *et al.*, 2001). Temozolomide, an oral formulation of dacarbazine with central nervous system (CNS) penetration, has recently been approved by the FDA for treatment of high-grade gliomas. Studies of temozolomide in advanced melanoma suggest a systemic response rate comparable to that seen with dacarbazine (15% to 20%) (Agarwala *et al.*, 2000; Middleton *et al.*, 2000). The role of temozolomide is currently being evaluated in clinical trials for the prevention and treatment of melanoma CNS metastases (O'Day *et al.*, 2002).

1.2.2.2 Combination Chemotherapy

The disappointing results of single-agent chemotherapy led to the development of combination regimens in the 1980s in efforts to improve outcome in patients with metastatic disease (O'Day *et al.*, 2002). Initial combinations with either a vinca alkaloid or cisplatin and dacarbazine showed minimal improvement in response rate (20% to 30%) (Lotze *et al.*, 2001). More aggressive multi-agent combinations have followed and resulted in response rates of up to 30% to 50% in single-institution phase II trials. Two published regimens, the Dartmouth regimen (carmustine, cisplatin, dacarbazine and tamoxifen) (Del Prete *et al.*, 1984) and CVD (cisplatin, vinblastine and dacarbazine) (Legha *et al.*, 1989), are the most commonly used as standard treatment for metastatic disease (O'Day *et al.*, 2002).

A phase III trial comparing CVD to dacarbazine alone showed a trend towards improved response rate and survival in the combination chemotherapy arm. However, these results were not statistically significant (Buzaid *et al.*, 1993). Chapman and co-workers published the results of a phase III trial comparing the

Dartmouth regimen to dacarbazine alone and also found that despite a trend towards an improved response rate (18.5 % vs 10.2 %) in the combination chemotherapy arm, the results were not statistically significant (Chapman *et al.*, 1999).

1.2.2.3 Tamoxifen Therapy

The effect of tamoxifen for the treatment of advanced melanoma has been the subject of controversy. It stems back to when melanoma cells were recognised to express oestrogen receptors. This led to a series of clinical trials with anti-oestrogen therapy. A European tamoxifen trial demonstrated a disappointing 5% objective response rate (Rumke *et al.*, 1992). Following its failure as a single agent tamoxifen was then tested in combination with chemotherapy. Cocconi and co-workers published a small phase III trial demonstrating an improved response (28% vs 12%, $p=0.03$) and overall survival (48 weeks vs 29 weeks, $p=0.02$) with the addition of tamoxifen to dacarbazine compared to dacarbazine alone (Cocconi *et al.*, 1992).

McClay *et al.*, (1989 and 1992) reported a significant reduction in the objective response rate when tamoxifen was omitted from the combination chemotherapy Dartmouth regimen in a series of small phase II trials. Pre clinical models suggested that high-dose tamoxifen synergised with cisplatin and reversed multidrug resistance (MDR) (McClay *et al.*, 1992; 1993; 1996, Desai *et al.*, 1995; Kirk *et al.* 1994; Cabot *et al.*, 1996). However, randomised trials by Falkson *et al.* (1998) and Rusthoven *et al.* (1996) with low and high-dose tamoxifen in combination with either dacarbazine or the Dartmouth regimen failed to demonstrate an advantage with the use of tamoxifen. This was confirmed by another phase II study of high-dose tamoxifen added to concurrent biochemotherapy. This study also failed to show improved response rates or survival (O'Day *et al.*, 2001).

Therefore, despite a promising start for antiestrogen therapy in melanoma, preclinical data and encouraging early clinical data, these findings have not been confirmed in subsequent phase II trials for the treatment of metastatic melanoma (O'Day *et al.*, 2002).

1.2.2.4 Isolated Limb Perfusion

Isolated limb perfusion chemotherapy is a method of giving very high doses of chemotherapeutic agents to treat metastatic tumours at surgically isolated local sites. This is particularly attractive compared with other chemotherapeutic regimens as it allows powerful agents to be given at much higher doses than is possible when they are administered systemically. One example of a chemotherapeutic drug used for isolated limb perfusion is Melphalan, an alkylating agent.

Melphalan induces complete remission in 35-40% of cases and partial remission in a further 35-40%. Melphalan has also been tested with TNF- α and IFN- γ . TNF- α combined with melphalan for isolated limb perfusion has yielded an overall response of 95% in melanoma (Lienard *et al.*, 1999). Eggermont and co-workers (1996) also recommended the use of isolated limb perfusion with TNF- α for sarcomas, as it was safe and an effective induction treatment with a high response rate.

1.2.3 Interferons and Hematopoietic Cytokines

Adjuvant therapies were developed from studies that showed some success in treating advanced melanoma. The principle assumption with this approach is that treatments that are effective against macroscopic melanoma will provide even greater benefit in eradicating micrometastatic disease, thus preventing future recurrence and ultimately death (Gray *et al.*, 2002). The use of biologic response modifiers for the treatment of melanoma has led to slight increases in the five-year survival of patients. The main cytokines in use together with surgery are IL-2 and Interferon alpha (IFN- α). Interferon has direct antiproliferative effects on melanoma cells and indirect effects through modulating the host immune response (Kirkwood *et al.*, 1996).

1.2.3.1 Low Dose Interferon Alpha

Single-agent IFN- α has a response rate of 15% and complete remission rates of 3% to 5% in advanced disease (Marincola *et al.*, 1995). Inman and co-workers (2003) reported a study in which low-dose adjuvant IFN- α 2b was administered to stage III melanoma patients. This study failed to show any statistically significant difference between low dose interferon adjuvant therapy and surgery alone. Interferon added to

dacarbazine in a small phase II trial by Falkson *et al.* resulted in an encouraging response rate of 53%. However, a follow-up large randomised trial demonstrated no benefit for the addition of interferon to dacarbazine (Falkson *et al.*, 1998).

Cascinelli and colleagues reported the findings of a trial carried out on patients with stage III melanoma. They were also unable to show any correlation with low dose interferon alpha and disease free or overall survival (Cascinelli *et al.*, 2001).

1.2.3.2 High Dose Interferon Alpha

High-dose interferon is the only biological treatment approved by the FDA for the adjuvant therapy of melanoma. However, its efficacy in this setting is questionable and its administration is associated with considerable toxicity.

Early trials using low-dose and intermediate-dose IFN regimens demonstrated no benefit to survival. However, the Eastern Cooperative Oncology Group trial EST 1684 showed that a high-dose regimen led to significant improvements in both disease-free and overall survival (Sabel *et al.*, 2003). This was the first adjuvant therapy to show significant survival benefit in melanoma patients ($p = 0.0023$). Table 1.7 shows a comparison of the 5-year survival of patients with cutaneous melanoma observed with surgery alone and with adjuvant interferon therapy. The response to therapy was greatest among patients with clinical evidence of nodal metastasis (stage III). The toxicity of IFN is significant (Kim *et al.*, 2002) with approximately 78% of patients treated experiencing grade 3 or greater toxicity (Kirkwood *et al.*, 1996; 1997).

On the basis of these results, the US FDA approved high-dose IFN- α 2b for post-surgical adjuvant therapy of high-risk melanoma. Unfortunately, the results of subsequent trials on high-dose IFN- α 2b therapy have not been as clear and its role in the adjuvant treatment of melanoma remains controversial (Sabel *et al.*, 2003).

In conclusion, low dose interferon therapy does not prolong survival in patients following surgery. On the other hand, high dose interferon has at least in some studies been shown to increase survival and therefore should be considered in future trials. However, the side effects associated with these therapies are a limiting factor.

AJCC stage	Surgery alone (Baseline prognosis)	Adjuvant interferon
IA	95%	96%
IB	90%	93%
IIA	77%	83%
IIB	65%	74%
IIC	45%	57%
IIIA	67%	76%
IIIB	53%	64%
IIIC	26%	39%

Table 1.7. Five-year overall survival of patients with cutaneous melanoma grouped by the American Joint Committee on Cancer (AJCC) with surgery alone and with surgery plus high dose adjuvant interferon therapy (Adapted from Thome *et al.*, 2002).

1.2.3.3 Interferon Gamma

IFN- γ has been shown to upregulate MHC class I and II expression and to promote generation of specific antitumour immune responses. Khorana and colleagues published the results of a study in 2003 that investigated the effects of immunotherapy with interferon-gamma (IFN- γ). This was a phase I dose-escalation study of a replication-deficient adenovirus interferon-gamma construct to determine safety and tolerability of intratumoural administration in advanced or locally recurrent melanoma. Correlative studies failed to reveal any evidence of immunological activity.

1.2.3.4 Granulocyte-Macrophage Colony-Stimulating Factor

A number of studies have investigated the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF) in clinical trials (Slingsluff *et al.*, 2003; Dillman *et al.*, 2003). Spitler *et al.* (2000) reported the results of a phase II trial evaluating GM-CSF as adjuvant therapy in patients with stage III or IV malignant melanoma. Patients were treated with long-term, chronic, intermittent GM-CSF after surgical resection of disease. Both overall and disease-free survival was significantly prolonged. The median survival was 37.5 months in patients who received GM-CSF compared with

12.2 months in matched historical controls (Spitler *et al.*, 2000). In addition, no significant toxicity was observed.

1.2.3.5 Interleukin 2

Keilholz and colleagues (1997) investigated the effect of interferon and IL-2 with or without cisplatin chemotherapy. Response rates were significantly increased from 18% to 33% with cisplatin. The median survival was similar in both groups. A larger phase III trial by the EORTC (Keilholz *et al.*, 1999) compared cisplatin dacarbazine, and interferon with or without decrescendo IL-2. In a preliminary analysis response rates were similar in both groups (22% vs 28%).

Another study by Rosenberg and colleagues (1999) performed a randomised trial of sequential dacarbazine, cisplatin, tamoxifen, interferon and high-dose bolus IL-2 compared to the chemotherapy alone. No phase II data had been generated with this biochemotherapy regimen prior to phase III testing. Considerable toxicity was observed in patients. The response rates were higher in the biochemotherapy arm (44% vs 27%). However, surprisingly there was a survival advantage in the chemotherapy-alone group (5.8 vs 10.7 months). In conclusion, with the exception of GM-CSF most trials failed to demonstrate an impact on disease-free survival and overall survival by cytokine treatment with or without chemotherapy (Eggermont, *et al.*, 2003).

1.2.4 Radiation Therapy

The efficacy of radiotherapy is limited as melanomas are less sensitive to ionising radiation than other tumours. It has been speculated that melanin may have a protective role by scavenging free radicals produced by ionising radiation (Hubbard-Smith *et al.*, 1992). Regional recurrence remains a problem in the management of patients with metastatic malignant melanoma in the cervical lymph nodes and parotid. O'Brian and colleagues (1997) conducted a non-randomised trial on the number of positive nodes, extra capsular spread and the use of adjuvant radiotherapy and its impact on regional control and survival. The authors concluded adjuvant radiotherapy was associated with (non-statistically significant) improved control of metastatic malignant melanoma in the neck and parotid. This and similar clinical studies

(reviewed by Cooper, 1998) shows that patients with some forms of melanoma can benefit from radiotherapy.

1.2.5 Cancer Specific Vaccines

William Coley administered the first cancer vaccine in the 1890s by injecting live bacteria to treat patients. A review of 30 patients treated with Coley's toxin (acute staphylococcal infection) showed that the neoplasms regressed following treatment and in a few cases the cancer disappeared completely without recurrence (Nauts, 1953).

The concept of the vaccine is derived from the practice of immunising against infectious agents to prevent disease. For vaccination against viral infections, individuals are immunised with viral antigens before encountering the pathogenic organism. This strategy became successful for immunisation against viruses because viral genes encode a limited number of defined antigens. However, in the case of most tumours, many more antigens can be the targets of the immune response. In addition, it is likely that the expression of many of these antigens is activated during or as a result of tumourigenesis (Jaffee *et al.*, 2000).

The enhanced understanding of the molecular mechanisms of lymphocyte activation and the identification of specific molecules mediating the immune response such as cytokines has led to a new era in vaccine design and development. Secondly, the more efficient transfer of genes encoding immunologically active molecules into tumour cells has led to the development of new and advanced strategies for vaccine construction.

At present the identity of many tumour antigens against which the immune response is directed, remains unknown for most cancers. Until the molecular structures of these antigens are identified, the tumour itself remains an attractive source of immunogen (Jaffee *et al.*, 2000). Due to its immunogenic nature melanoma has frequently been used as a tumour model for vaccine construction (Muehlbauer *et al.*, 2003).

1.2.6 Melanoma Vaccines

Morton and colleagues (1970) were the first to demonstrate complete regression of metastatic melanoma nodules after intralesional injection of live BCG. Ninety percent of melanoma metastases regressed. Many clinical trials have confirmed this observation (Hanna *et al.*, 1973; Mujagic *et al.*, 1979; Orefice *et al.*, 1978). The induction of systemic antitumour immunity by BCG is suggested by (a) regression of non-injected nodules at sites distant from intralesional injections, (b) the relationship between the clinical course of disease and the *in vitro* cytotoxicity of lymphocytes in the presence of autologous serum, and (c) the regression of a pulmonary metastasis in a patient treated for multiple intradermal metastases (Mastrangelo *et al.*, 1976).

Current melanoma vaccines in both preclinical and clinical stage include ganglioside vaccines, recombinant protein vaccines, protein vaccines, anti-idiotypic vaccines and polyvalent shed antigen vaccines. These are described in further detail below. These vaccines are reasonably well characterised, elicit reproducible responses and do not induce anti- HLA class I and II antibodies.

1.2.6.1 Ganglioside Vaccines

Livingston and colleagues (1985) reported a study on patients immunised with tumour cell vaccines consisting of irradiated autologous melanoma cells or allogeneic melanoma cell lines mixed with adjuvants such as BCG, *C. parvum*, vesicular stomatitis virus and treated with neuraminidase, trypsin or glutaraldehyde. The authors found that humoral responses were often directed against HLA, viral antigens or bovine proteins used in the culture of melanoma cells. However, the melanoma-associated gangliosides GM2 and GD2 were found to be immunogenic (Livingston *et al.*, 1985). This finding led to a randomised clinical trial of purified GM2 that concluded that the overall survival benefit for the GM2-BCG group was insignificant (Livingston *et al.*, 1991; 1993).

The immunogenicity of gangliosides coupled to KLH and administered with QS-21 adjuvant was tested in melanoma patients (Helling *et al.*, 1993; 1994). Tumour cells expressing the targeted ganglioside were eliminated initially but residual tumour cells with no or low levels of ganglioside expression emerged and became resistant to

therapy. Therefore, the heterogeneity of melanoma-associated gangliosides can be a major hindrance to the success of therapies with ganglioside antigens.

Portoukalian and colleagues (1991) administered an autoclaved polyvalent purified melanoma ganglioside vaccine. IgM and IgG antibody titres against all the melanoma gangliosides were assessed and significantly fewer recurrences found in patients without elevated IgG antibody titres. The median disease-free interval was 71 weeks for responders compared with 26 weeks for non-responders.

1.2.6.2 Anti-idiotypic Antibody Vaccines

Anti-idiotypic antibodies (anti-ids) are considered as potent melanoma vaccines since they mimic melanoma-associated antigens (MAAs). Extensive studies in animal models have demonstrated the efficacy of these vaccines for triggering the immune system to induce specific and protective immunity against bacterial, viral and parasitic infections as well as tumours (Bhattacharya-Chatterjee *et al.* 2001). Ferrone *et al.* (1993) administered monoclonal anti-id antibodies mimicking an epitope of the HMW-MAA intradermally to AJCC stage IV melanoma patients. Disease progression was observed in 10 of 24 patients (Ferrone *et al.*, 1993). Foon *et al.* (1998) reported a study of 12 patients with advanced malignant melanoma treated with an anti-idiotypic antibody that mimics the disialoganglioside GD2. Lymphocytes isolated from five patients mediated antibody-dependent cellular cytotoxicity *ex vivo*. One patient had a complete clinical response and the remaining six patients had stable disease ranging from 9 to 23 months (Foon *et al.*, 1998).

Alfonso *et al.* (2002) generated an anti-idiotypic mAb (Ab2) to an Ab1 mAb that reacts specifically with N-glycolyl-containing gangliosides, antigens expressed by human melanoma and breast carcinoma cells. The authors tested the Ab2 in a clinical trial with 20 patients with advanced malignant melanoma. Sixteen of the 17 patients who received at least four doses of the anti-Id vaccine developed Ab3 antibodies capable of inhibiting Ab2 binding to Ab1. In addition, a very strong Ab3 response against N-glycolyl-containing gangliosides was induced in these 16 patients. No adverse effects were observed but clinical response was not reported (Alfonso *et al.*, 2002).

1.2.6.3 Adoptive Transfer Therapy

Partial or complete tumour regression has been induced by adoptive transfer of T cells derived from tumour-infiltrating lymphocytes (TILs) and cultured with IL-2. Other studies have shown massive infiltration of CD4⁺ and CD8⁺ lymphocytes in regressing tumours from patients receiving IL-2- mediated immunotherapies and accumulation of infused T lymphocytes labelled with ¹¹¹Indium in tumour sites (Rubin *et al.*, 1989; Fisher *et al.*, 1989). T lymphocytes isolated from TILs and peripheral blood lymphocytes from melanoma patients are able to recognise autologous and allogeneic melanoma cells that share class I MHC alleles (Darrow *et al.*, 1989). Antibodies to MHC class I molecules can prevent lysis of melanoma cells by CD8⁺ T cells. Transfection of class I MHC genes (such as human leukocyte antigen A2 [HLA-A2]) into melanoma cells not expressing the MHC class I allele renders these cells susceptible to lysis by CD8⁺ lymphocytes (Wolfel *et al.*, 1989; O'Neil *et al.*, 1993). In addition, CD4⁺ lymphocytes from TILs recognise melanoma antigens presented by MHC class II molecules. However, *ex vivo* manipulation of TILs is difficult and prevents the routine use of TIL therapy with current methodology.

1.2.6.4 Purified Protein and Peptide Vaccines

Purified protein or peptide vaccines are aimed at stimulating T lymphocytes *in vivo* to cause tumour regression (Ravindranath *et al.*, 1999). Current antigen peptide trials in progress include a phase II randomised study of immunisation with an HLA-A2 multiepitope peptide vaccine comprised of MART-1, gp100 and tyrosinase derived peptides alone or in combination with GM-CSF and/or interferon alfa-2b in HLA-A2 positive patients with metastatic melanoma. A phase III randomised study of GM-CSF and peptide vaccination comprised of tyrosinase, gp100 antigen and MART-1 peptides versus peptide vaccination alone, GM-CSF or versus placebo in patients with locally advanced or metastatic melanoma is also being conducted (National Cancer Institute, www.cancer.gov).

1.2.6.5 Shed Antigen Vaccines

Polyvalency could increase the potential of a vaccine to stimulate protective immunity. Melanoma cells in culture release almost half of the material expressed on

their external surface within 3 hours but release only a fraction of their internal molecules. The shed material consists of cell-surface proteins and other macromolecules that can be purified (Bystryn *et al.*, 1986). Bystryn and co-workers (1986) harvested polyvalent shed antigens from three human and one hamster melanoma cell line, purified these antigens, depleted their HLA component and then tested the HLA-depleted antigens in a phase I clinical trial. The authors observed complete regression of cutaneous metastases in one patient (1 of 13), who showed no evidence of disease for more than 60 months.

A subsequent study showed that the vaccine stimulated both humoral and cellular immune responses to melanoma in approximately 50% of 94 patients (AJCC stage III) with surgically resected regional disease (Bystryn *et al.*, 1992). The authors reported a correlation between anti-melanoma cellular immune responses and favourable clinical outcome. Median disease free survival was 4.7 years and overall survival was 3.7 years longer in patients that made a strong DTH response to the vaccine. Furthermore, 70% of patients with a strong DTH response were still disease free 3 years after treatment, compared with 31% of nonresponders. The ability of shed melanoma antigen to slow disease progression has since been confirmed in a phase III patient trial (Bystryn *et al.*, 2001).

1.2.6.6 Cell Lysate Vaccines

The concept of viral and bacterial oncolysate melanoma vaccines is based on the principle that antitumour immune responses can be augmented by adding a viral or bacterial derived foreign component to vaccines.

This was tested in a clinical trial investigating whether immunotherapy with a vaccine prepared from vaccinia melanoma cell lysates would improve relapse-free survival and overall survival in 700 patients with stage IIB or III melanoma. The authors observed a trend towards improved survival in the vaccine group. However, this was not statistically significant (Hersey *et al.*, 2002).

1.2.6.7 Viral Melanoma Oncolysate

Wallack and colleagues (1977) used vaccinia virus to prepare a viral melanoma oncolysate (VMO) from cell lines established from four patients with primary and metastatic melanoma. Most VMO recipients developed DTH reactions. In addition, patients who were negative for anti-MAA antibodies before treatment developed antibodies after vaccine treatment (Wallack *et al.*, 1977). However, a subsequent phase III randomised trial of VMO versus vaccinia virus in 215 patients with high-risk stage II melanoma failed to show a significant difference in disease-free interval or overall survival (Wallack *et al.*, 1995).

Berthier-Vergne and colleagues showed that lymphocytes from melanoma patients responded more strongly than lymphocytes from normal individuals *in vitro* to VMO stimulation in the presence of low concentrations of IL-2. Post vaccination sera of patients had increased levels of IgG to a 31 kDa antigen normally detectable in tumour metastases (Berthier-Vergne *et al.*, 1994). These findings suggest a possible therapeutic benefit of VMO vaccine in human melanoma and were in accordance with the observations made by Savage and colleagues (1986). The authors reported antibody development in six melanoma patients following 6 weeks of immunisation with allogeneic melanoma oncolysates prepared from three Newcastle virus-infected melanoma cell lines.

However, a later study by Hersey and colleagues (2002) reported the results of a phase III study with 700 patients that found immunotherapy with viral melanoma lysate was not associated with a statistically significant improvement in overall, or relapse free survival (Hersey *et al.* 2002).

1.2.7 Gene Therapy

In gene therapy foreign genes are introduced into either tumour cells or the host immune cells. The goal of gene therapy targeted to melanoma cells is the introduction of suicide genes or transfer of tumour suppressor genes to inactivate aberrant oncogene expression or to introduce genes encoding immunologically active molecules, such as co-stimulatory molecules and/or cytokines (Sotomayor *et al.*, 2002). Table 1.8 lists examples of the gene therapy strategies currently under

investigation for melanoma. Gene therapy targeted at the host immune cells, i.e. melanoma-infiltrating lymphocytes or dendritic cells, have been developed as an additional strategy to redirect immune responses against melanoma.

Successful development of gene therapy is dependent on two key elements. Firstly, the identification of critical genes that are associated with a missing biological function leading to a specific disease and secondly, an effective way to deliver these genes to specific target cells.

Gene Therapy Targeted to Melanoma Cells

Introduction of "suicide" genes

- herpes simplex virus thymidine kinase gene (HSV tk)

Transfer of tumour suppressor genes

- p53 gene
- p16INK4a

Inactivation of oncogenic signalling pathways

- *ras*
- *c-myc*
- signal transducers and activators of transcription factor-3 (Stat3)

Introduction of genes encoding immune activating molecules

- allogeneic MHC class I genes
- cytokine genes (GM-CSF, IL-2, IFNs, etc.)
- co-stimulatory molecules (B7.1)

Gene Therapy Targeted to Host Immune Cells

T cells

- neomycin phosphotransferase gene
- chimeric receptor (IL-2R/GM-CSFR)

Dendritic cells

- genes encoding melanoma antigens (MART-1/Melan A)
- CD40 ligand

Table 1.8: Examples of gene therapy targeted to melanoma cells and to host immune cells (adapted from Sotomayor et al., 2002).

1.2.7.1 Introduction of Suicide Genes into Melanoma

The herpes simplex virus thymidine kinase (HSVtk) gene is the most widely used suicide gene for melanoma gene therapy in clinical trials. This gene is introduced into tumour cells and patients are subsequently given the drug ganciclovir. Ganciclovir is an acyclic nucleoside analogue that, when phosphorylated by HSVtk, is incorporated into DNA resulting in the termination of DNA elongation during the S-phase of transduced tumour cells (Reid *et al.*, 1988). The human thymidine kinase has a low affinity for ganciclovir and therefore this drug has little toxicity in humans (Gane *et al.*, 1997).

Klatzmann and colleagues (1998) reported phase I/II studies using herpes simplex virus type 1 thymidine kinase as suicide gene therapy for metastatic melanoma. HSV-1tk expression sensitised transduced and bystander cancer cells to ganciclovir. Patients received one to three injections of retrovirus vector-producing cells into cutaneous melanoma nodules. Treatment-related adverse events were mild and transient. The transgene was detected in three of six patients tested. The tumour size in the treated tumours was moderately reduced in comparison with untreated tumours and necrosis of treated tumours was detectable by histology in three of eight patients. The authors concluded that despite a good tolerance the responses to the treatment were limited due to poor gene transfer efficiency *in vivo*.

1.2.7.2 Transfer of Tumour Suppressor Genes

p53 gene is the most frequently mutated tumour suppressor gene in human cancers and is the most common target for corrective gene therapy used in clinical trials (Levine, 1997). Although point mutations of p53 are rare in melanoma, transfection and overexpression of this gene in melanoma cells results in apoptosis of tumour cells expressing mutated p53 and containing the transfected wild-type allele (Schneeberger *et al.*, 2000).

A phase I dose escalation study of single intratumoral (i.t.) injection of a replication-defective adenoviral expression vector containing wt p53 was tested in patients with metastatic melanoma and breast cancer. Patients with increased p53 protein immunoreactivity in pre-treatment tumour biopsies were selected for the study. Five

of six patients tested became positive for the wt p53 gene 2 days after injection. All six patients developed anti-adenoviral antibodies with mild adverse reactions (Dummer *et al.*, 2000). This study provides evidence to suggest that p53 gene therapy using a replication-defective adenoviral expression vector is safe, feasible and biologically effective in patients with either metastatic melanoma or breast cancer. Clinical responses were not reported.

1.2.7.3 Blockade of Oncogenic Signaling Pathways

Activation of oncogenes frequently occurs in melanoma and contributes to the malignant phenotype (Castellano *et al.*, 1999). Therefore these oncogenes (such as members of the *ras* family and *c-myc* as well as mutated tumour suppressor genes) represent potential targets for the gene therapy of melanoma. Several approaches have been studied to reduce the expression of activated oncogenes. One option is to introduce a gene coding for a ribozyme. A ribozyme is RNA with catalytic activity and cleaves mRNA resulting in reduced expression of the oncogene (Arndt *et al.*, 1997). Another method of inactivating oncogenes is the introduction of a gene that codes for a scFv antibody that is specific for the onco-gene product. When expressed within the tumour cells, the scFv will bind and thus inactivate the onco-gene product. An example of this strategy was published by Cochet *et al.* (1998) who showed that expression of an anti-Ras scFv resulted in specific inhibition of the Ras signaling pathway in *Xenopus laevis* oocytes and NIH3T3 fibroblasts. In addition, they showed that neutralising Ras with the scFv specifically promoted apoptosis *in vitro* in transformed human cancer cells (Cochet *et al.*, 1998).

Signal transducers and activators of transcription (STATs) are transcription factors that function as key mediators of cytokine and growth factor signalling pathways (Darness *et al.*, 1997). In addition to control of cell proliferation, differentiation and apoptosis, numerous studies have demonstrated that constitutively activated STAT signalling, particularly Stat3, directly contributes to oncogenesis and malignant progression in human cancers (Bowman *et al.*, 2000). Gene therapy of B16 melanoma with a dominant-negative, inactive signal transducer and activator of transcription Stat3 variant resulted in inhibition of tumour growth and tumour regression *in vivo* (Niu *et al.*, 1999).

1.2.7.4 Transfection with genes encoding Cytokines or Co-Stimulatory Molecules

Effective gene transfer techniques have enabled the genetic modification of melanoma cells to secrete cytokines locally or to express new and increased levels of cell-surface molecules. Using this approach the immunogenicity of melanoma cells can be increased by enhancing the presentation of tumour antigens and/or by providing co-stimulatory signals to the T-cell arm of the immune system (Pardoll 1993).

In preclinical models, these strategies prime systemic immune responses capable of rejecting a subsequent tumour challenge or eradicating established micrometastatic tumours. An example of this strategy is priming with GM-CSF-transduced tumour cells. Priming with GM-CSF leads to a potent, long-lived antitumour immunity that requires the participation of both CD4⁺ and CD8⁺ T cells (Chong *et al.*, 1998; Heller *et al.*, 2000). Further investigation of the mechanisms mediating this strong anti-tumour effect showed that local GM-CSF secretion by melanoma promotes both recruitment and activation of the host antigen-presenting cells that uptake, process and present tumour antigens to antigen-specific T cells leading to strong antitumour responses (Huang *et al.*, 1994).

Soiffer *et al.*, reported the findings of a clinical trial vaccinating patients with autologous lethally irradiated melanoma cells engineered to produce human GM-CSF. Vaccines were successfully made for 97% of patients (35 total). Ten patients were alive and four patients showed no evidence of disease after 3 year follow up (Soiffer *et al.*, 2003).

Custom-made vaccines are very expensive and their preparation is laborious. Therefore, simpler approaches need to be explored. Borrello *et al.* (1999) used a mixture of autologous tumour cells and MHC-negative allogeneic GM-CSF-producing bystander cells to vaccinate mice. This resulted in an antitumour immune response equivalent to or better than when autologous tumour cells directly transduced to secrete GM-CSF were used (Borrello *et al.*, 1999).

Plautz *et al.* (1993) injected allogeneic MHC class I encoding plasmid DNA admixed with cationic lipids into tumours. The authors observed significantly delayed tumour growth and found that animals were resistant to subsequent challenge with the wild-type tumour. This was subsequently tested in a phase I clinical trial (Nabel *et al.*, 1993). No apparent toxicity was observed and all patients evaluated generated anti melanoma cytotoxic T lymphocytes (CTLs). In addition, regression of a treated lesion as well as uninjected melanoma nodules was observed in one of 5 patients (Nabel *et al.*, 1993).

1.2.7.5 Genetically modified Immune Cells

T cells recognising autologous tumour have been isolated from vaccinated animals and melanoma patients (Mukherji *et al.*, 1990; Topalian *et al.*, 1994). Adoptive transfer studies showing melanoma regression in experimental models demonstrated antitumour activity of tumour reactive T cells (Melief *et al.*, 1995). Rosenberg and co-workers (1990) published results of the first human gene transfer study in cancer patients evaluating the expression of the neomycin phosphotransferase gene in TILs. In addition to exhibiting an anti-tumour effect in some patients, the feasibility and safety of this gene transfer approach targeting immune effector cells was also shown (Rosenberg *et al.*, 1990). However, despite initial optimism the low response rates, short response duration and significant toxicities associated with the simultaneous use of high doses of IL-2 have limited the enthusiasm for adoptive transfer of TILs in melanoma patients (Maria *et al.*, 2002). Yee *et al.* used a retroviral vector encoding a chimeric IL-2 receptor with the extracellular domain of GM-CSF receptor to render CD8⁺ T cells helper-independent, to avoid the need of exogenous IL-2 administration for *in vivo* therapy (Yee *et al.*, 1997).

Growing knowledge of the central role of APCs in the initiation of immune responses has led to dendritic cell (DC)-based gene transfer strategies being investigated in experimental models and melanoma patients (Maria *et al.*, 2002). DCs are potent APCs capable of initiating effective T cell responses (Banchereau *et al.*, 1998). These cells generally express 50-fold higher levels of MHC molecules than macrophages and provide more peptide/MHC ligands for T-cell receptor engagement. DCs also express extremely high levels of co-stimulatory and adhesion molecules critical for T-

cell activation. The development of *in vitro* culture techniques allowing the generation of large numbers of DCs have made these cells an attractive target for gene therapy strategies (Inaba *et al.*, 1992).

Strategies to genetically modify DCs and enhance their therapeutic efficacy include liposomal transfection, gene gun or viral transfer of genes encoding well-defined tumour-associated antigens and co-stimulatory molecules (Maria *et al.*, 2002). Song *et al.* (1997) and Specht *et al.* (1997) showed that murine DCs genetically modified to express beta-galactosidase generated strong antitumour responses in cancer vaccination models with β -gal transfected murine tumour cell lines. Reeves *et al.* (1996) demonstrated the feasibility of transducing human DCs with the gene encoding the melanoma antigen MART-1. These DCs elicited a strong antigen-specific CTL response and triggered enhanced cytokine production by MART-1-specific TIL. Furthermore, Ribas *et al.* (1997) showed that immunisation with DCs transduced with a MART-1/Melan-A encoding recombinant adenoviral vector resulted in the induction of strong anti-melanoma immunity superior to strategies using DNA or adenovirus vaccination against MART-1 (Grewal *et al.*, 1996). Kikuchi and co-authors (2000) investigated the role of CD40 ligand/CD40 interaction in the initiation of antigen-specific T-cell responses and in the prevention of tumour induced T-cell unresponsiveness. CD40 ligand (CD40L) is expressed on activated T_H cells. Its interaction with CD40 molecules on DCs leads to DC activation (upregulation of B7 and ICAM-1 expression and release of IL-12). The authors observed that intratumoural administration of CD40L transduced DCs elicited tumour-specific cytotoxic T-lymphocyte responses, and the transfer of spleen cells from CD40L-DC-treated mice efficiently protected naive mice against a subsequent tumour challenge. Sotomayor *et al.* investigated the effect of genetically modified DCs *ex vivo* with a recombinant CD40L adenovirus vector. Intratumoural injection of CD40L-modified DCs into B16 melanoma nodules resulted in tumour regression and survival advantage (Sotomayor *et al.*, 1999).

Martin *et al.* (1999) fused the single-chain Fv antibody fragment (scFv) LHM2 directed against high-molecular-weight melanoma-associated antigen (HMW-MAA) to the murine leukaemia virus envelope protein. The modified viruses bound to HMW-MAA-expressing cells. Following attachment to HMW-MAA, MMP cleavage

of the envelope at the melanoma cell surface removed the scFv and proline-rich hinge, allowing infection. Complexing of targeted retroviruses with liposomes increased their efficacy without affecting their target cell specificity (Martin *et al.*, 1998; 1999). A subsequent study showed melanoma specific retargeting of the modified reterovirus *in vivo* (Martin *et al.*, 2002). Nettelbeck *et al.* (2004) described another example of gene retargeting to melanoma with RAFT3 scFv and an adenovirus vector.

In conclusion, the initial enthusiasm generated by successful gene transfer in preclinical models has been dampened by the lack of major successes in recently completed clinical trials. However, limited positive results obtained have shown the safety and feasibility of this approach. The early clinical studies have also unveiled the limitations and obstacles that need to be overcome in order to make gene therapy an effective treatment modality for malignant melanoma.

1.3 ANTIBODIES

The basic structural unit of most mammalian antibodies is a glycoprotein with a molecular weight of approximately 150 kDa. Immunoglobulins are composed of four polypeptide chains, two light chains and two heavy chains connected by disulphide bonds (Figure 1.2). The light chain has a molecular weight of approximately 25 kDa and is composed of one variable domain (V_L) and one constant domain (C_L). There are two types of light chains, lambda (λ) and kappa (κ). In humans, 60% of the light chains are κ , and 40% are λ , whereas in mice, 95% of the light chains are κ and only 5% are λ . A single antibody molecule contains either κ or λ light chains.

Each heavy chain has a molecular weight of approximately 50 kDa and consists of a constant and variable region. The heavy and light chains contain a number of homologous domains with similar structures. These homologous units consist of about 110 amino acids and are called immunoglobulin domains. The heavy chain consists of one variable domain (V_H) and either three or four constant domains (C_{H1} , C_{H2} , C_{H3} and C_{H4}) depending on the antibody class or isotype. The C_{H1} and C_{H2} domains are connected through a hinge region. It enables flexible movement of the two Fab arms of the antibody molecule thus allowing them to open and close so that the antibody can bind to two antigenic determinants simultaneously. The basic structure of an antibody molecule is illustrated in Figure 1.2.

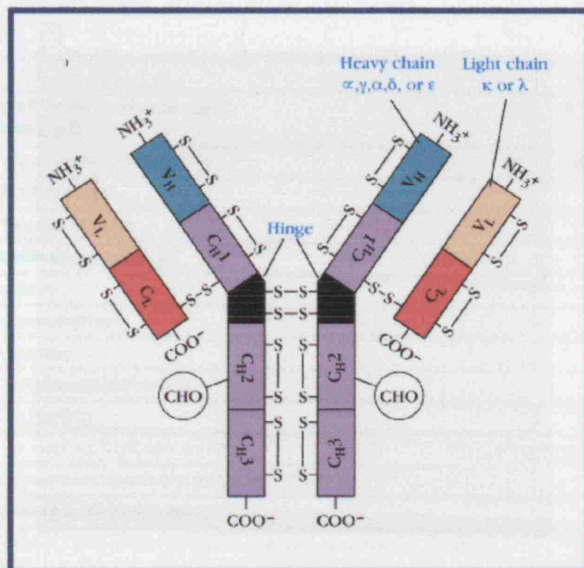


Figure 1.2: 2D Antibody structure. The basic components of immunoglobulin G are shown. The disulphide bonds of the immunoglobulin are indicated (adapted from Kuby, 2000).

The heavy chain determines the functional activity of the antibody molecule. The five antibody classes IgG, IgA, IgM, IgE and IgD differ by their heavy chains (γ , α , μ , ϵ and δ , respectively). Whilst the IgD, IgE and IgG antibody classes consist of a single structural unit, IgA antibodies contain either one or two units whereas IgM antibodies consist of five disulfide-linked structural units. The biological properties of the 4 human IgG subclasses are illustrated in Table 1.9.

The Fc region is glycosylated and has 2 main effector functions (binding complement, binding to cell receptors on macrophages and monocytes, etc.) and distinguishes different antibody classes. The complement system is discussed in detail in Chapter 3 Section 3.1.7 - 3.1.15

	IgG1	IgG2	IgG3	IgG4
Adult serum level range (g/l) (mean, g/l)	4.9-11.4 (6.98)	1.5-6.4 (3.8)	0.20-1.10 (0.51)	0.08-1.40 (0.56)
Proportion of total IgG (%)	43-75	16-48	1.7-7.5	0.8-11.7
Half-life (days)	21	21	7	21
Placental transfer	+	+	+	+
Antibody response to:				
proteins	++	+/-	++	+/-
polysaccharides	+	++	(-)	(-)
Allergens	+	(-)	(-)	++
Complement activation				
C1q binding	++	+	+++	-
C1q binding, high epitope density	++	++	+++	-
enhancement alternative pathway	-	+	-	+/-
Binding to Fcγ receptors:				
FcγRI (CD64) monocytes, macrophages, neutrophils, dendritic cells	++	-	+++	+
FcγRII (CD32) monocytes, macrophages, neutrophils, eosinophils, platelets, B cells, dendritic cells, endothelial cells	++	(a)	+++	-
FcγRIIa-H131	++	+++	+++	-
FcγRIIa-R131	++	-	++	-
FcγRIII (CD16) neutrophils, eosinophils, macrophages, NK cells, subsets of T cells	++	-	++	-
FcγRIIIb-NA1	+++	-	+++	-
FcγRIIIb-NA2	++	-	++	-
Binding to Staphylococcal protein A	++	++	(b)	+
Binding to Streptococcal protein G	++	++	++	++
Functional valency	2	2	2	1

Table 1.9: Biological properties of human IgG subclasses (<http://www.xs4all.nl/~ednieuw/IgGsubclasses/subkl23.htm>). (a): FcγRII allotype dependent ; (b): IgG₃ allotype dependent.

1.3.1 Monoclonal Antibodies

In contrast to conventional cytotoxic chemotherapy and radiation therapy antibody therapy is directed against cancer associated antigens that form part of molecular pathways leading to a malignant phenotype. These therapies target specific tumour cell receptors or signalling molecules that are critical to tumour progression while reducing toxicity to normal cells (Kim *et al.*, 2002).

In the 1980s monoclonal antibodies promised an era of ‘magic’ bullet therapies that could specifically kill tumour cells while leaving healthy tissues undamaged. The specificity of monoclonal antibodies (Mab) was predicted to make them ideal weapons in the battle against cancer. However, subsequent studies sometimes showed severe limitations of their use *in vivo*. Most of the Mabs used in humans have

been generated in mice and thus induce human anti-mouse antibodies (HAMA) (Mountain *et al.*, 1992). This can result in accelerated clearance of the administered Mab and hence lower the efficacy of treatment. More recently, it has been shown that antibody immunogenicity can be reduced in preclinical models, e.g. by chemical modification with PEG but more fundamental problems still remain (Pedley *et al.*, 1994). Humanised monoclonal antibodies should be more effective in patients than mouse mAbs.

1.3.2 Monoclonal Antibody Formation

Monoclonal antibodies are produced from B cell clones that are specific for a single epitope. The B cells are fused with myeloma cells to generate B cell/myeloma hybrids (hybridomas). Myeloma cells are malignant plasma cells therefore, hybridomas possess the immortal growth properties of the myeloma cells and are able to secrete the antibody produced by the B cells. The resulting hybridoma cells secrete large quantities of the monoclonal antibody and can be cultured indefinitely (Figure 1.3).

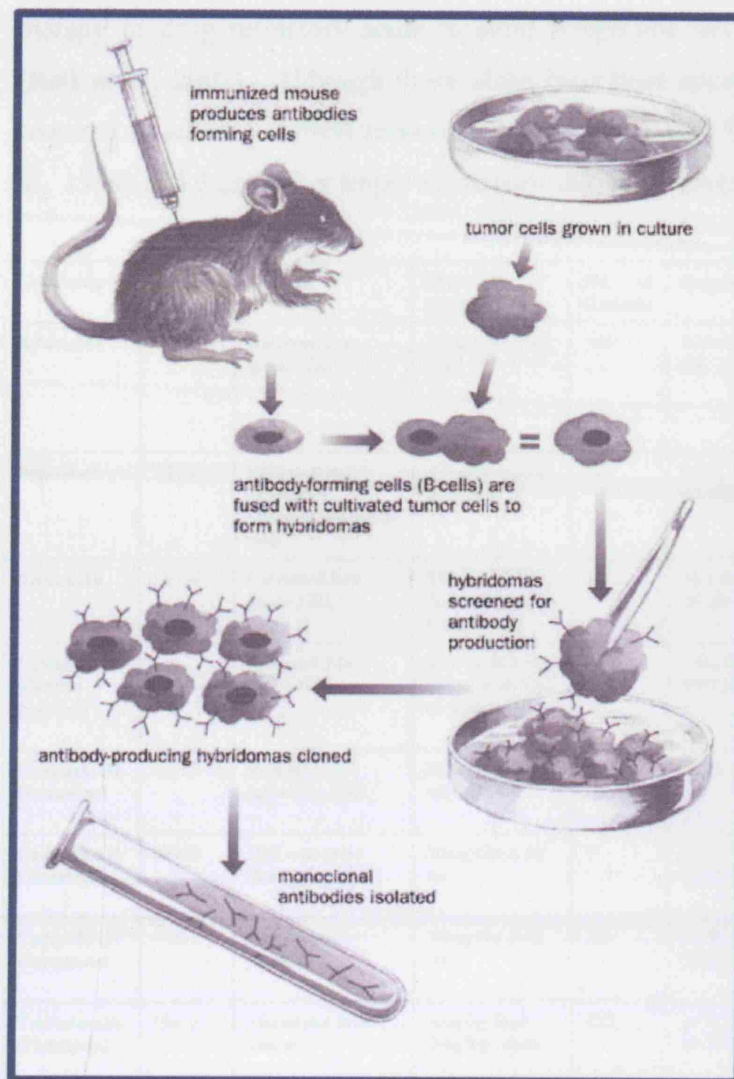


Figure 1.3: Production of monoclonal antibodies (<http://www.nap.edu/books/0309057779/html/83.html>).

1.3.3 Monoclonal Antibody Therapy

Some human or partially human monoclonal antibodies that have been tested in patients include Rituxan, Campath-1H and Mylotarg. Rituxan (rituximab) is a recombinant chimeric anti CD20 monoclonal antibody used for the treatment of non-Hodgkin's lymphoma that was approved in 1997 in the United States. It was the first monoclonal antibody to receive FDA approval. This paved the way for the use of monoclonal antibodies as therapeutic agents. The US Food and Administration (FDA) has recently approved alemtuzumab (Campath-1H), a humanised rat antibody to CD52 for the treatment of refractory chronic lymphocytic lymphoma. Gemtuzumab ozogamicin (Mylotarg), a calicheamicin conjugated humanised anti-CD33 Mab for

therapy of drug refractory acute myeloid lymphoma has also been FDA approved (Reff *et al.*, 2002). Although these Mabs have been approved for therapy the doses required to achieve clinical responses are very high (see Table 1.10) (Goldenberg *et al.*, 1999) and thus further improvements by antibody modification are required.

Antibody	Target	Disease	Dosing regimen	No. of Patients	Response	Problems	Study
Rituximab	CD20	Relapsed low grade NHL	375mg/m ² q wk x 4	204	50% CR + PR	Mild infusional toxicity Rare CD20 escape, Rare HACA	Maloney <i>et al.</i> , 1997 McLaughlin <i>et al.</i> , 1998
Rituximab	CD20	Relapsed and refractory intermediate grade NHL	375-500 mg/m ² q wk x 8	54	5 CR 12 PR	Mild infusional toxicity	Coiffer <i>et al.</i> , 1998.
Rituximab	CD20	Untreated low grade NHL	375mg/m ² 6 doses – 6 cycles CHOP	38	22 CR 16 PR	Mild infusional toxicity No added toxicity to CHOP	Czuczman <i>et al.</i> , 1999.
Custom anti-idiotypic (murine)	Ig idiotype	Relapsed low grade NHL	400-11,500 mg alone, with Ch1 or with INF	34	18% CR 50% PR	Mild infusional toxicity Id negative escape	Meeker <i>et al.</i> , 1985 Brown <i>et al.</i> , 1989
Campath-1H Humanised	CD52	Relapsed and refractory NHL	30mg tiw x 12 wk	42	6 PR	Moderate infusional toxicity, Immunosuppression	Lundin <i>et al.</i> , 1998
Campath-1H Humanised	CD52	CLI – no prior therapy	30mg tiw x 18 wk	9	3 CR 5 PR	Moderate infusional toxicity, Immunosuppression	Osterborg <i>et al.</i> , 1996
Campath-1H Humanised	CD52	CLI – prior therapy	30mg tiw x 12 wk	29	1 CR 11 PR	Moderate infusional toxicity, Immunosuppression	Osterborg <i>et al.</i> , 1997
Trastuzumab (Herceptin)	Her-2	Metastatic breast cancer	4mg/kg load 2mg/kg/ week	222	8 CR 26 PR	Mild toxicity, cardiac toxicity	Cobleigh <i>et al.</i> , 1999
Trastuzumab	Her-2	Metastatic breast cancer	4mg/kg load 2mg/kg/ week with AC	146	36-62% PR	18% cardiac toxicity	Shak, 1999 (Randomised trial)
Trastuzumab	Her-2	Metastatic breast cancer	4mg/kg load 2mg/kg/ week with paclitaxel	89	25 – 75% PR	2% cardiac toxicity	Shak, 1999 (Randomised trial)
Edrecolomab 17-1A	Ep-Cam	Resected Duke-s C colon cancer	500 mg then 100 mg x 4	189	Decreased distant mets	Approved in Europe, confirmatory trials ongoing	Riethmuller <i>et al.</i> , 1998 (randomised trial)

Table 1.10: Examples of some promising unconjugated monoclonal antibodies in clinical trials. Tiw, twice weekly; q, four times weekly; NHL, non hodgkins lymphoma; CLI: chronic lymphocytic leukaemia; ALI: acute lymphoblastic leukaemia; AML: acute myelogenous leukaemia; CR: complete response; PR: partial response; HACA: human anti chimeric antibody immune response; CHOP: cyclophosphamide, doxorubicin, vincristine and prednisone; Ch1: chlorambucil; AC: anthracycline and cyclophosphamide (adapted from Maloney, 2000).

1.3.3.1 Conjugated Monoclonal Antibodies

Spitler *et al.* predicted that unmodified monoclonal antibodies would not benefit patients with solid tumours (Spitler *et al.*, 1987). Conjugated monoclonal antibodies can be used to target cytotoxic agents to tumour cells for direct killing. This approach would have the advantage of increasing selective tumouricidal activity and decreasing the systemic toxicity of these cytotoxic agents (Spitler *et al.*, 1987). An example of this approach is the development of immunotoxins by coupling monoclonal antibodies to bacterial toxins that inhibit ribosomal functions.

Ricin A is an enzyme that inhibits protein synthesis by ribosomal inactivation, thereby causing cell death (Olsnes and Pihl, 1973). A single molecule of ricin A chain in the cytosol can cause cell death. In therapy with immunotoxins the monoclonal antibody binds specifically to melanoma cells and the toxin kills the cells to which the antibody is bound. A phase II trial has been conducted with an anti-melanoma antibody conjugated to ricin A (XomaZyme-Mel). Gonzalez and co-workers published a study of 20 patients with metastatic melanoma treated with the murine antibody immunotoxin. As expected, the treatment had significant toxicities. Two patients had a clinical response with one complete response (Gonzalez *et al.*, 1991).

Gemtuzumab ozogamicin [Mylotarg] is a chemotherapeutic agent consisting of a humanised anti-CD33 antibody linked to calicheamicin, a potent enediyne antitumour antibiotic. The Federal Drug Administration approved Mylotarg in May 2000 as single-agent therapy for first recurrence of acute myeloid leukemia (AML) in older patients after a clinical trial showed a complete response rate of 20% (Table 1.11; Sievers *et al.*, 1999). It has also been used as maintenance therapy in patients with AML and acute promyelocytic leukaemia (Giles *et al.*, 2003).

Antibody	Target	Toxin or Drug	Disease	Dose (MTD))	No. of Patients	Response	Problems/ comments	Study
CMA-676	CD33	Calicheamicin	Relapsed or refractory AML	9mg/m ² x2	40	8 CR	Post infusion chills fever, rigors. Pancytopenia until recovery	Sievers <i>et al.</i> , 1999
Anti-B4 (Murine)	CD19	Blocked ricin	Refractory ALL, CLL, NHL	1-60µg/kg/d x 5d (50µg/kg/d)	25	1 CR 2 PR	Transaminasesemia, fever, HAMA, HARA	Grossbard <i>et al.</i> , 1992
Anti-B4 (Murine)	CD19	Blocked ricin	Refractory ALL, CLL, NHL	CI 10-70µg/kg/d x 7 d (50µg/kg/d)	34	2 CR 3 PR	Transaminasesemia, fever, thrombocytopenia, HARA	Grossbard <i>et al.</i> , 1993
Anti-B4 (Murine)	CD19	Blocked ricin	NHL after ABMT	CI 20-50µg/kg/d x 7 D q 28d x 2 (40µg/kg/d)	12	11 CR 13-26 mo after ABMT	Transaminasesemia, fever, thrombocytopenia, HARA	Grossbard <i>et al.</i> , 1993
HD37 (Murine)	CD19	DgA	Refractory low grade and intermediate grade NHL	Bolus 2-24mg/m ² /8d (16mg/m ² /8d) CI 9.6-19.2mg/kg/8d	23 9	1 CR 1 PR 1 PR	Vascular leak syndrome, aphasia, acrocyanosis, HAMA, HARA	Stone <i>et al.</i> , 1996
Anti-B4 (Murine)	CD19	Blocked ricin	AIDS related NHL	CI 20µg/kg/d x 7d 2 cycles + m-BACOD 8 cycles	44	13 CR 12 PR	Vascular leak syndrome, aphasia, acrocyanosis, HAMA, HARA	Scadden <i>et al.</i> , 1998
Fab'-FRB4 (Murine)	CD22	DgA	Refractory NHL	25-120 mg/m ³ (75 mg/m ³)	15	38% PR	Vascular leak syndrome, myalgia, fever, aphasia	Vitetta <i>et al.</i> , 1991
IgG-FRB4 (Murine)	CD22	DgA	Refractory NHL	5-142 mg/m ³ (32 mg/m ³)	26	1 CR 5 PR	Vascular leak syndrome, myalgia, fever, aphasia	Amlot <i>et al.</i> , 1993
IgG-FRB4 (Murine)	CD22	DgA	Refractory NHL	9.6-28.8mg/m ³ (19 mg/m ³)	18	4 PR	Vascular leak syndrome, myalgia, fever	Sausville <i>et al.</i> , 1995

Table 1.11: Examples of selected Immunotoxins and conjugated monoclonal antibodies. NHL: non-Hodgkin's lymphoma; CLL: chronic lymphocytic leukaemia; ALL: acute lymphoblastic leukaemia; AML: acute myelogenous leukaemia; CR: complete response; PR: partial response; PBSC: peripheral blood stem cells; ABMT: autologous bone marrow transplantation; HAMA: human anti-murine antibody immune response; HARA: human anti-ricin antibody (adapted from Maloney, 2000).

1.3.3.2 Radiolabelled Monoclonal Antibodies

Ibritumomab tiuxetan consists of the murine anti-CD20 chimeric monoclonal antibody rituximab that has been linked covalently to the metal chelator MD-diethylenetriamine pentaacetic acid (DTPA) enabling stable binding of ¹¹¹Indium when used for radionuclide tumour imaging and ⁹⁰Y titanium when used for therapy (Krasner *et al.*, 2001; Wagner *et al.*, 2002). Ibritumomab tiuxetan became the first radioconjugated antibody therapeutic for cancer approved by the FDA in 1992. A phase 3 trial comparing ibritumomab tiuxetan with a standard dose of rituximab in patients with NHL showed that the overall response rate was 80% in the ⁹⁰Y

ibritumomab treated patients compared with 56% in the rituximab arm of the study (Krasner *et al.*, 2001).

¹³¹I-labelled anti-p97 Fab fragments for radiotherapy are examples of a monoclonal antibody that has been used for targeted melanoma therapy (Larson *et al.*, 1983). The study with 33 melanoma patients demonstrated localisation and tumour uptake of Fab fragment specific for p97, an oncofoetal glycoprotein. Blood clearance of p97-specific Fab was significantly more rapid than of non-specific Fab (¹²⁵I labelled) but no clinical responses were observed. At present there have not been any successful clinical trials with the radiolabelled mAbs in melanoma patients.

Antibody	Target	Disease	Isotope	Dose (mCi)	No. of Patients	Response	Problems/ comments	Study
B1 (Murine)	CD20	Relapsed low and intermediate grade NHL	¹³¹ I	34-161	28	14 CR 8 PR	Minimal infusional toxicity, myelosuppression	Kaminski <i>et al.</i> , 1993
Y2BB or B1 (Murine)	CD20	Relapsed low and intermediate grade NHL	⁹⁰ Y	14-53	18	6 CR 7 PR	myelosuppression requiring PBSC support, infection	Knox <i>et al.</i> , 1996
B1 (Murine)	CD20	Relapsed NHL	¹³¹ I	280-785	29	23 CR 2 PR	With AMBT support, reversible cardiopulmonary toxicity	Liu <i>et al.</i> , 1998
Lym-1 (Murine)	HLA-DR	Relapsed diffuse large cell NHL	¹³¹ I	37-384	25	3 CR 10 PR	Mild infusional toxicity HAMA	Lewis <i>et al.</i> , 1995
Lym-1 (Murine)	HLA-DR	Refractory NHL	¹³¹ I	52-290	21	7 CR 4 PR	Myelosuppression HAMA	Denardo <i>et al.</i> , 1998
Lym-1 (Murine)	HLA-DR	Refractory CLI	¹³¹ I	160-500	5	1 CR 4 PR	Thrombocytopenia	Denardo <i>et al.</i> , 1994
BC8	CD45	Relapsed ALI or AML	¹³¹ I	10.5	34	10 relapse free	Autologous or allogeneic BMT	Mathews <i>et al.</i> , 1999
				3.5-5.5	21	18 alive and relapse free	With HLA- matching sibling	

Table 1.12: Examples of selected promising clinical trials with radiolabeled monoclonal antibodies. NHL: non Hodgkin's lymphoma, CLI: chronic lymphocytic leukaemia, ALI: acute lymphoblastic leukaemia, AML: acute myelogenous leukaemia, CR: complete response, PR: partial response, PBSC: peripheral blood stem cells, AMBT: autologous bone marrow transplantation, HAMA: human anti murine antibody immune response (adapted from Maloney 2000).

1.3.3.3 Problems with the Clinical Use of Monoclonal Antibodies

Despite initial promises of great success the use of monoclonal antibodies has shown shortcomings due to their large size that impedes tumour penetration *in vivo* (Yokota *et al.*, 1992) and difficulties to produce antibodies at large scale. The future clinical success of monoclonal antibody based cancer diagnosis and therapy is dependent on

solving pharmacokinetic and delivery problems. These include slow elimination of monoclonal antibodies from the blood, poor vascular permeability, low and heterogeneous tumour uptake, cross-reactivity with normal tissues, metabolism of monoclonal antibody conjugates, the immunogenicity of murine antibodies in humans (Reilly *et al.*, 1995) and toxic side effects have been a major obstacle in the development of therapeutic antibodies for cancer (Carter *et al.*, 2001; Goldenberg *et al.*, 2002; Reichert *et al.*, 2001; 2002). The generation of an anti-antibody immune response depends on many factors. These include the dose of antibody, number of antibody injections, inherent immunogenicity of the antibody, form of the antibody and the immunocompetence of the recipient (Kuus-Reichel *et al.*, 1994).

1.3.3.4 Genetic Engineering of Monoclonal Antibodies

With the development of genetic engineering techniques it is now possible to generate mouse/human chimeric antibodies composed of mouse derived antigen binding variable regions and human derived constant regions (Boulianne *et al.*, 1984). Since the clinical application of mouse monoclonal antibodies is hampered by the induction of a HAMA (human anti mouse antibody) response, humanised, chimeric or human antibodies are preferable (Juweid *et al.*, 1995) and have now been tested in clinical models. Generally, humanisation abolishes the HAMA response against the Fc part but only in approximately 50% of mAbs against the V region. T_H epitopes generated in CDRs can be removed by ‘deimmunisation’ (Mateo *et al.* 2000) to reduce the immunogenicity of mouse/human chimeric antibodies.

1.3.3.5 Reduction of the HAMA Response

Murray *et al.* (1994) reported that an increased whole body clearance rate correlated with elevated human anti-mouse antibody in a phase II radioimmunotherapy trial with ¹³¹I-CC49 Mab in colorectal cancer. CC49 Mab specifically recognises a tumour-associated glycoprotein (TAG-72) expressed by a majority of human carcinomas. A recent study by Gonzales *et al.*, (2003) illustrates that the HAMA response can be modified by genetic engineering. The authors attempted to minimise the HAMA response by constructing two versions of humanised CC49 (HuCC49) by generating an array of scFv variants and replacing the murine framework residues retained in the humanised Ab with their counterparts in a human template by site-specific

mutagenesis. The variants were tested for their Ag-binding activity and reactivity with sera from patients previously administered with murine CC49 mAb. One variant showed a significant decrease in its reactivity to the anti-variable region Abs present in the patients' sera (Tamura *et al.*, 2000; Kashmiri *et al.*, 2001; Gonzales *et al.*, 2003). This identified one immunogenic position in the murine V domain.

Forero *et al.* (2003) also reported an attempt to reduce the immunogenicity of radiolabelled antibodies. The authors generated a complementary-determining region (CDR) grafted humanised antibody (MAb) and tested tumour localisation and immunogenicity in a pilot patient trial. The authors found that the antibody was only weakly immunogenic. However, the effect of repeated administration was not tested (Forero *et al.*, 2003).

1.3.4 Review Antibody Engineering

The first reports of protein engineering were made in the 1980s with the development of site directed mutagenesis. This technique was first used to mutate genes encoding enzymes with known mechanisms of action to produce mutant proteins with defined amino-acid residue substitutions. Among the first reported were tyrosyl-transfer RNA synthetase (Winter *et al.*, 1982) and β – lactamase (Sigal *et al.*, 1982). The modification of one or two amino acids has since been extended to replacements of entire loops (Jones *et al.*, 1986) and domains (Neuberger *et al.*, 1984).

A major problem with the clinical use of monoclonal antibodies is that their large size results in poor tumour penetration and therefore the core of the tumour escapes from antibody mediated therapy. An early application of antibody engineering to antibodies was the generation of antibody fragments as recombinant proteins in the periplasm of *E. coli* (Better *et al.*, 1988; Huston *et al.*, 1988; Skerra and Pluckthun, 1988). This permits efficient production and provides a more rapid and convenient method of engineering antibodies for specific functions. Phage display was another major breakthrough in the field of antibody engineering providing rapid and efficient selection of genetically engineered antibodies (McCafferty *et al.*, 1990; Rader and Barbas, 1997; Hoogenboom *et al.*, 1998). Hawkins *et al.* (1992) described a process, based on display of antibodies on the surface of filamentous bacteriophage, for

selecting antibodies either by their affinity for antigen or by their kinetics of dissociation (off-rate) from antigen. The authors showed that the relative increase in affinity of the mutant antibody is comparable to the increase seen in a murine secondary immune response (Hawkins *et al.*, 1992).

Jones *et al.* (1986) investigated the possibility of transplanting antigen binding sites from one framework to another by grafting the CDRs. The authors reported that 'CDR replacement' allowed construction of human monoclonal antibodies from the corresponding mouse monoclonal antibodies. Antibodies made by this approach have elicited a considerably reduced immune response, with promising clinical responses (Hale *et al.*, 1988), in comparison with murine antibodies and antibodies developed by chimerisation. Other studies have described the process of reshaping an antibody by grafting antigen binding sites from non-human antibodies into human framework regions (Jones *et al.*, 1986; Winter *et al.*, 1993; Jones *et al.*, 1986; Riechmann *et al.*, 1988; Verhoeyen *et al.*, 1988). However, the clinical experience with humanised antibodies was not as promising as anticipated. This is reviewed in more detail in Section 1.1.23.

Resurfacing is a process in which variable domains from mouse antibodies can be resurfaced by replacing the surface residues in the mouse framework region with human residues (Roguska *et al.*, 1994; 1996).

A further development to improve the pharmacokinetics and biodistribution of monoclonal antibodies and improve tumour penetration was the development of small single chain Fv fragments (scFvs) that penetrate the tumour much more efficiently while retaining the targeting properties of whole antibodies. The single chain Fv fragment is covalently connected via a peptide linker connecting the C-terminus of V_H or V_L with the N-terminus of the other domain (Huston *et al.*, 1988; Plückthun *et al.*, 1991). A general problem associated with the use of scFv fragments was stability. This issue was addressed by the inclusion of a stabilising disulphide bond at the interface of the two domains (Glockshuber *et al.*, 1990; Brinkman *et al.*, 1993; Reiter *et al.*, 1996). Antibody fragments fused to peptides have been shown to form homodimeric (Pack and Plückthun, 1992) and heterodimeric (Kostelny *et al.*, 1992) antibody fragments that improve the avidity of the artificial antibody multimers.

Likewise, antibody fragments have been engineered for improved solubility and stability (Plückthun *et al.*, 1991).

Their faster clearance due to the small size of scFv fragments (29 kDa) makes them tumour-imaging reagents superior to monoclonal antibodies. ScFvs offer increased tumour to normal tissue ratio, but they have a lower absolute uptake in the tumour than whole IgG molecules. This is because they are small monovalent molecules and quickly cleared from the blood via the kidneys. This issue has been addressed by making scFvs divalent without causing a significant increase in their plasma half-life. An example of such a study is the construction of a covalently dimerised anti c-erbB-2 scFvs by engineering a cysteine residue at the C-terminal end of the scFv (Adams *et al.*, 1993). This study demonstrated that a dimerised scFv exhibits enhanced tumour retention due to divalent binding, despite being the same size as a Fab fragment.

In vivo studies using single chain Fv fragments have demonstrated that multimers with two or more binding sites have higher apparent affinity in addition to an improved avidity and thus are an ideal option for tumour targeting (Hu *et al.*, 1999; Adams and Schier *et al.*, 1999; Viti *et al.*, 1999; Hudson and Kortt, 1999).

Bi-functional chelating agents have also utilised in combination with an engineered cysteine residue at the C-terminus of scFv to both dimerise the scFv and for site specific radiolabelling (Olafsen *et al.*, 2004). The valency of scFvs has been increased by shortening the linker between the two V domains to 4 amino acids to form divalent and bivalent scFvs. This forces the V domains to form inter-molecular complexes with their cognate domains (Holliger *et al.*, 1993). A recent study by Santimaria *et al.* (2003) has illustrated the high efficacy for imaging of radiolabelled diabodies of the anti-ED-B domain antibody in patients.

ScFvs have been further modified to improve their valency by constructing minibodies. Minibodies are scFvs fused to the C_H3 domain of IgG resulting in non-covalent dimerization. Compared with scFvs, minibodies have better tumour targeting efficiency with short half-life (Wu *et al.*, 1996).

To improve the efficacy of antibody therapy a method of delivering cytokines to the tumour environment has been developed. Antibodies that are specific for tumour-associated antigens can increase the effective concentration of cytokines in the tumour microenvironment, if the antibodies are genetically fused to cytokines. For example, the antibody fusion protein of chimeric IgG₁ fused to human GM-CSF has shown promising results for the treatment of B cell malignancies in mice (Hornick *et al.*, 1997). Huston *et al.* (1993a and 1993b) have described the utility of genetic fusions between effector proteins and toxins as useful tools in medicine and diagnostics. Fusion proteins are discussed in further detail in Sections 1.3.41-1.3.4.3.

Antibody fragments have also been adapted to deliver drugs to the target antigen by a number of successive steps. This approach has been dubbed ADEPT (Bagshawe *et al.*, 1988). Antibody fragments are conjugated or fused to different enzymes that are able to convert an inactive prodrug to an active drug. Examples of enzymes used include β -lactamase, glucuronidase, alkaline phosphatase and carboxypeptidase. This is discussed in further detail in Section 4.1.12.

Single chain Fv fragments have been used successfully as fusion to toxin proteins. These consist of an antibody fragment genetically linked to a toxin molecule. The mechanism of action allows antibodies to target the tumour cell, whilst the fused toxin internalizes and kills the target cell. The toxin binds to the cell surface, translocates to the cytosol, causes enzymatic inactivation of e.g. protein synthesis (Allured *et al.*, 1986; Katzin *et al.*, 1991; Rutenber and Robertus, 1991; Choe *et al.*, 1992) and induces apoptosis. Antibody fragments fused to the C terminus of DT immunotoxins have been explored (Chaudhary *et al.*, 1990; 1991). A recombinant PE derived immunotoxin is the anti-Tac (Fv) PE38 for the treatment of B and T cell lymphoma and leukemias (Chaudhary *et al.*, 1989; Kreitman and Pastan, 1995). Mansfield *et al.* (1997) studied a dsFv specific for CD22 fused with PE38 and observed that antitumour activity was dose responsive in nude mice. Some recombinant immunotoxins have been tested in clinical trials and shown improved anti-tumour activity (reviewed by Li *et al.*, 2005).

Bispecific antibodies directed against an antigen on the tumour cell and an antigen on the effector cells have also been developed. These are discussed in Section 5.1.5.1.

The first bispecific antibody to enter clinical trials was shown to be highly effective in the re-direction against CD3 and CD19 on lymphoma cells (Loffler *et al.*, 2000).

1.3.5 ScFv Fragments

More recently molecular engineering has allowed the construction of single chain Fv fragments (scFv). In scFvs the variable heavy and light chain gene segments are cloned with a synthetic peptide linker (Winter *et al.*, 1994). This results in a 28kDa single chain antibody fragment that retains antigen binding ability.

Single chain Fvs are cleared more quickly than mAbs thus making them ideal candidates for diagnostic tumour imaging (Colcher *et al.*, 1989). Therapeutic versions of single chain Fvs are alternatives for immunotherapy compared with conventional monoclonal and bispecific antibodies. Their smaller size permits increased tumour penetration by scFv and this may allow more effective tumour destruction. They can also be easier to produce (Schultz *et al.*, 2000). Furthermore, it has been suggested that human antibodies, chimeric antibodies and mouse Fab fragments are less likely to induce anti-antibody responses than intact mouse monoclonal antibodies or mouse F(ab')₂ fragments, when only a single injection is administered (Kuus-Reichel *et al.*, 1994).

1.3.5.1 ScFv Toxins and SEA Fusion Proteins

Recombinant immunotoxins consist of Fv regions of cancer specific antibodies fused to truncated bacterial toxins. Many recombinant immunotoxins contain a truncated version of *Pseudomonas* exotoxin as a toxic moiety. The recombinant antibody fragments will target the modified toxin to cancer cells that are then killed. The affinity of immunotoxins and their ability to enter and penetrate into tissues and tumours is dependent on the size of the protein that needs to be optimised (Brinkmann *et al.*, 2000).

Ueno *et al.* (2002) fused SEA, the bacterial superantigen staphylococcal enterotoxin A, a potent activator of CD4⁺ T cells to a scFv. The fusion protein binds MHC class II constant domains and bypasses MHC presentation. The scFv recognises MK-1, a glycoprotein antigen present on most carcinomas including melanoma. The SEA

scFv fusion protein retained the reactivity with MK-1-expressing tumour cells, mediated specific cytotoxicity of lymphokine-activated killer T-cells to the tumour cells and suppressed tumour growth in a SCID mouse xenograft model.

Tordsson *et al.* (2000) selected several clones producing scFv antibody fragments that reacted with HMW-MAA by phage selection of a recombinant antibody phage library. One scFv fragment (K305) was selected, transferred and expressed as a Fab-fragment fused to the superantigen staphylococcal enterotoxin A. The fusion protein redirected T cell cytotoxicity to melanoma cells *in vitro* and reduced tumour growth *in vivo* (Tordsson *et al.*, 2000).

Dohlsten *et al.* (1994) published another example of SEA fusion proteins by constructing a recombinant fusion protein of SEA and the Fab region of the C215 monoclonal antibody specific for human colon carcinoma cells. The FabC215-SEA fusion protein targeted T cells to lyse C215⁺ MHC class II⁺ human colon carcinoma cells. Treatment of immunocompetent mice grafted with C215 transfected mouse melanoma resulted in 85-99% tumour growth inhibition and subsequently long-term survival of animals. The results of this study provide evidence for Fab-SEA fusion proteins specifically retargeting the immune response to the tumour cells by stimulation of T cells.

1.3.5.2 MHC scFv Fusion Molecules

Down-regulation of the major histocompatibility complex (MHC) is one of the major mechanisms that tumour cells use to escape immunosurveillance. Li *et al.* (2003) hypothesised that specifically coating tumour cells with foreign MHC will make tumour cells a better target for immune recognition and surveillance. The authors generated a fusion protein, H2K^d/scPSMA, consisting of a single chain Fv against human prostate specific membrane antigen (PSMA) and the extracellular domain of mouse H-2K^d. The results showed that expression of H-2K^d/scPSMA in a B16F10 cell line expressing human PSMA and transfected with the gene encoding the fusion protein significantly inhibited tumour growth and improved overall survival (Li *et al.*, 2003).

1.3.5.3 Chimeric scFv Fusion Proteins

Myers *et al.* (2002) constructed chimeric proteins linking either the human IgG₁ Fc domain or the extracellular domain of murine B7.1 to a scFv specific for the oncofoetal glycoprotein 5T4 expressed by carcinomas. The fusion proteins interact simultaneously with both 5T4-positive cells and the ligands of the immune effector moieties (FcγRs or CTLA-4). The scFv-Fc fusion protein directed lysis of 5T4-expressing human tumour cell lines through antibody-dependent cellular cytotoxicity indicating its potential as a therapy for human cancers (Myers *et al.*, 2002).

Gerstmayer *et al.* (1997) demonstrated costimulation of T cell proliferation by a chimeric B7-2 antibody fusion protein specifically targeted to cells expressing the *erbB2* proto-oncogene. The B7-2 anti-ErbB2 fusion protein localises specifically to the surface of ErbB2-expressing target cells providing costimulatory signals that enhance proliferation of syngeneic T cells (Gerstmayer *et al.*, 1997). These results suggest potential for targeting chimeric ligands to the surface of tumour cells as potential tumour vaccines for cancer immunotherapy.

1.3.6 Design of ScFv Fragments Used in This Study

The experience of clinical trials with humanised antibodies (reviewed in Section 1.1.23) clearly indicated that further modifications were required to antibodies to optimise them for therapy. One such approach was to make smaller fragments that would penetrate the tumour more effectively, have improved pharmacokinetic and biodistribution properties and be more stable and with reduced side effects. As discussed above, this was attempted by making single chain Fv fragments (reviewed in Section 1.1.23.5). Single chain fragments are promising molecules due to their small size and properties as discussed earlier. For these reasons the scFv RAFT 3 was chosen for fusion with protein A molecules.

The RAFT3 single chain Fv used for these studies was derived from the LHM2 monoclonal antibody and has been characterised and studied (*in vitro* and *in vivo*) as a potential modality for the radioimaging of malignant melanoma (Kang *et al.*, 2000). The LHM2 scFv (London Hospital Medical College clone 2 scFv) was designed and constructed by Dr Jörg Kupsch (Kupsch *et al.*, 1995; 1999). It is directed against the

high molecular weight melanoma associated antigen (HMW-MAA). The antibody fragment was produced by isolating the V_L and V_H gene segments of the anti HMW-MAA mouse monoclonal antibody LHM2 (IgG₁). The V gene segments were cloned using mouse V region-specific PCR primers. The PCR fragments (approximately 350bp) were amplified with appropriate primers in a further PCR reaction to introduce a 15 amino acid linker and to assemble the two V segments using splicing by overlap extension (Horton *et al.*, 1989). This LHM2 cassette was then subcloned into plasmid pCantab5. The subcloned plasmid (encoding LHM2 scFv) includes an N-terminal secretion signal that directs transport of the scFv into the bacterial periplasm and a C-terminal c-myc sequence for detection by Western blot.

A human-mouse chimeric scFv library was designed and constructed by Dr. Jörg Kupsch by chain-shuffling. Antibody fragments with the same melanoma specificity as the original LHM2 scFv but differing human V_k segments were selected from that library and used for further analysis by our laboratory. Antibody phage display was employed to select phage expressing anti-melanoma scFv by panning the library against humans A375M melanoma cells. By retaining the original mouse V_H region specific for HMW-MAA, the specificity of the chimeric constructs for the same antigen was conserved. Phage display was employed for the selection of scFvs as it has the advantage of selecting clones with increased affinity for the antigen. The RAFT3 scFv is one such chimeric antibody fragment and was used to construct the fusion protein RAFT3ZZ.

1.3.7 HYPOTHESIS

Single chain Fv protein A fusions have the ability to recruit both mouse and human IgG, thus providing the opportunity to study immunotherapy in a realistic *in vivo* model. Their antigen binding specificity and smaller size would make them ideal candidates for molecules with increased tumour penetration.

Single chain Fvs fused to protein A also have the potential to be more potent than Mabs as they are able to bind 2 IgG molecules and all IgG isotypes. In addition, fusion of protein A could increase the immunogenicity of scFv and this could trigger an active anti-idiotypic response of potential therapeutic value.

1.3.8 AIMS

- I. To construct therapeutic scFv fusion molecules.
- II. To demonstrate that a single chain Fv fusion with two artificial domains of protein A (ZZ) recruits IgG *in vitro* and has the potential to re-direct melanoma specific kill.
- III. To test the therapeutic efficacy of the scFv protein A fusion molecule in a mouse model.
- IV. To optimise the dosing regimes for scFv fusion molecule treatment.
- V. To examine the side effects and immunogenicity of the fusion molecule and scFvs.

Following the unexpected observation that scFvs are highly immunogenic (Chapter 4) these original aims were extended to include:

- VI. To map the epitope(s) on scFvs that triggers an immune response.
- VII. To investigate the therapeutic potential of scFvs.
- VIII. To optimise the dosing regimes for treatment with unmodified scFv.

Chapter 2

Materials and Methods

2 MATERIALS AND METHODS

2.1 GENERAL MOLECULAR BIOLOGICAL TECHNIQUES

2.1.1 Plasmid Extraction

E. coli TOP10 (Invitrogen) was used as cloning and expression host. Plasmid extraction was carried out using the Wizard-Miniprep™ System (Promega).

A bacterial (1.5-2ml) overnight culture was centrifuged in a microcentrifuge to obtain a pellet (13,000rpm, 1min, room temperature (RT)). The pellet was resuspended in 200µl of buffered glucose solution. Two hundred µl of NaOH and SDS solution was added to lyse the bacteria. The cell lysis solution was neutralised using 200µl of buffer containing potassium acetate and acetic acid. The suspension was then centrifuged to remove bacterial debris (13000rpm, 10min, RT). The cleared supernatant was removed to a fresh tube and mixed with 1ml of DNA-binding resin. The sample was then loaded onto a 2ml mini-column and suction applied using a vacuum manifold. The resin/DNA mix was washed with 2ml of column wash solution (Tris-buffer plus 60% ethanol). Fifty µl of distilled H₂O was added and the column allowed to stand for 5 to 10min. The released DNA was then eluted from the column by centrifugation at 13000rpm for 1 min.

2.1.2 DNA Purification

DNA samples from restriction digests or PCR reactions were purified using agarose gel separation and Wizard PCR Purification Resin (Promega). Protein in the sample was denatured by heating to 68°C for 10 minutes in a heating block. The DNA sample was loaded onto a 1.5% TAE gel in 1x TAE buffer and separated at 100V for ~40 min. The band of interest was identified under UV light and cut out of the gel. The gel fragment was then heated in 1ml PCR Purification Resin at 65°C until melted and the gel was left at room temperature for 10 mins. The gel mix was then loaded onto a 2ml mini column and suction applied using a vacuum manifold. The DNA was washed with 80% isopropanole. The DNA was eluted from the column with 50µl of distilled water. Water was added to the column and allowed to stand for 10 min. The released DNA was then recovered from the column by centrifugation at 13000 rpm

for 1 min. The purity and yield of DNA was confirmed by loading an aliquot on a further 1.5% TAE gel.

2.1.3 Polymerase Chain Reaction (PCR)

Polymerase chain reactions were carried out according to established techniques (Molecular Cloning: A Laboratory Manual, 1989). Appropriate 3' and 5' primers were designed and obtained from MWG Biotech. Primer stocks (100 μ M) were made and brought to a concentration of 10 μ M prior to use.

One μ l DNA template (approximately 50ng), 10 μ l 3' oligonucleotide (10 μ M), 10 μ l 5' oligonucleotide (10 μ M), 10 μ l 10 x buffer, 16 μ l dNTPs (1.25mM each), 47 μ l distilled water, 5 μ l DMSO and 1.25 μ l DynAzyme (2.5 units) (Finnzymes) were mixed for the reaction. Two drops of mineral oil (Sigma Aldrich) were added to each reaction to prevent evaporation during thermocycling. Samples were then placed in a thermoblock (Biometra). A touch-down PCR program was used as follows.

The reaction was heated to 94 °C for one minute and the following steps carried out ten times: 1 minute at 94°C, 2 minutes at 55°C, 3 minutes at 72°C. Following the completion of the first cycle the temperature of step two is reduced by 1°C, i.e the temperature for step two becomes 54 °C for the following cycle. This was repeated for ten cycles until the temperature of step two reaches 45 °C. This samples the optimal annealing conditions for the PCR and hence reduces background while improving yield. Once the first ten cycles have been completed the following steps are carried out twenty times: 1 minute at 94°C, 2 minutes at 45°C, 3 minutes at 72°C. Finally, the PCR reaction is heated to 72 °C for 5 minutes and then kept at 4 °C.

2.1.4 Gel electrophoresis

All DNA electrophoresis was carried out in Tris-acetate EDTA (TAE) buffer following standard methods. All agarose gels were poured in 1x TAE buffer made from a stock solution of 50 x TAE. The stock solution was made as 243g/l Tris base (BDH), 20.5 g sodium acetate (water-free) brought to pH7.7 with glacial acetic acid

(BDH), 18.5g/l EDTA and made up to 1 litre with distilled water. The stock solution was diluted to 1 x TAE with distilled water.

Standard 0.8% or 1.5% agarose gels were made as 0.8g and 1.5g agarose powder (Gibco), respectively, 100ml 1xTAE buffer and 5.0µl ethidium bromide (Gibco). The percentage of gel used depended on the expected size of the DNA analysed. A 0.8 % gel was used for ≥ 0.65 kb whereas a 1.5 % gel was used for 0.2 – 1.2 kb DNA fragments.

The gel mixture was heated to melt in a microwave oven, cooled to 60 °C and poured into a mould with an appropriately sized comb. The gel was allowed to set for thirty minutes. Once the gel had set the comb was removed and the gel submerged in 1 x TAE buffer. The DNA samples were mixed with loading buffer and loaded onto the gel. A current of 110 V, 220 mA was applied for 40 minutes and the DNA electrophoresed towards the anode. The gel was then removed and viewed under ultra violet light to detect the DNA. The samples were electrophoresed with either a 1kb or a 123bp DNA size ladder depending upon the size of the fragment of interest.

2.1.5 Purification of vector by electro-elution

The digested vector was electrophoresed on a 0.8% TAE gel for purification. The correct size band was cut out and placed in a 5 cm piece of dialysis bag (0.2 µM pores) containing 500µl TAE buffer. The dialysis bag was clipped at both ends ensuring no air bubbles were trapped. The dialysis bag was then placed in an electrophoresis tank and submerged in TAE buffer. An electric current was applied and the DNA electrophoresed out of the gel into the buffer in the dialysis bag.

A Sephadex G50 mini column equilibrated with water was centrifuged for two minutes at 1800 rpm and the runthrough discarded. The buffer containing the eluted DNA was loaded onto the column. The column was centrifuged for four minutes at 2400 rpm. The DNA eluate was collected and ethanol precipitated.

2.1.6 Purification of PCR fragments

The PCR reaction was electrophoresed on a 1.5% TAE gel. The correct size band was cut out, placed in a microcentrifuge tube and 1 ml PCR purification resin (Promega) was added. The sample was heated for 5 minutes at 65 °C. Once the agarose had completely dissolved it was left at room temperature for 10 minutes whilst a purification column and a syringe were attached to a vacuum manifold. The contents of the microcentrifuge tube were transferred to the syringe. Once it had run through the syringe into the column, 2 ml 80 % isopropanole was added. The column was then dried for a further 10 minutes, removed from the vacuum and detached from the syringe. Fifty µl distilled water pre-heated to 68 °C was added to the column and allowed to stand for 1 minute. The purified DNA fragment was eluted by centrifugation of the column.

2.1.7 Restriction Digest

Restriction digests were carried out using appropriate restriction enzymes and buffers (Roche Biochemicals). DNA samples were prepared in dH₂O and enzyme and buffer were added. Reactions were carried out at temperatures appropriate for the particular enzyme until complete digestion was demonstrated following electrophoresis on a TAE gel. The reaction was terminated by heating the DNA to 68°C for 10 min.

2.1.8 DNA Ligation

Ligations were made at a molar ratio of 1 to 1 DNA fragment to vector. Ligations were made up to a maximum volume of 10 µl of which 1 µl was T4 DNA ligase (Roche Biochemicals) and 1 µl was 10 x ligase buffer (Roche Biochemicals). The ligation reaction was incubated overnight at 16 °C in a water bath.

2.1.9 Ethanol precipitation

Half a µl tRNA, 1/10th volume 3M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol were added to the DNA. The sample was incubated overnight at - 20°C. The following day the sample was centrifuged for 10 minutes at 13000 rpm. The supernatant was discarded, the DNA pellet washed with 75 % ethanol and centrifuged

for a further 10 minutes at 13000 rpm. The supernatant was discarded. The pellet dried in a vacuum for 30 minutes and then resuspended in distilled water.

2.1.10 Butanol precipitation

Ten or more volumes of butan-1-ol were added to the DNA. The sample was vortexed and then centrifuged for 10 minutes at 13000 rpm. The supernatant was discarded and the pellet washed with 75% ethanol. The sample was centrifuged for a further 10 minutes at 13 000 rpm and the supernatant was discarded. The pellet was dried in a vacuum for 30 minutes and then resuspended in distilled water.

2.1.11 Bacterial Culture Media and Agar Plates

Luria Broth base (Life Sciences) was used for all bacterial culture. Twenty five g of broth base was added per litre and autoclaved at 15psi, 120°C for >20min before use.

Agar plates were prepared using Luria Agar (Life Sciences) at 17g of agar per litre of distilled H₂O. The agar was sterilised by autoclaving as the broth above. The agar was melted in a conventional microwave oven and cooled to ~60°C in a water bath before adding glucose, IPTG or antibiotic as appropriate and poured immediately. The plates were allowed to set and stored at 4°C for up to two weeks. Before use, the plates were dried at 37°C for one hour.

2.1.12 Bacterial Transformation

During transformation by electroporation transient pores are formed in the membrane of competent *E.coli* and the DNA is taken up into the bacteria. Competent bacteria were stored at -80 °C in a medium (10% glycerol, 0.125% yeast extract and 0.25% tryptone) that gives good transformation efficiency without impairing bacterial survival.

Competent cells were taken out from storage at -80 °C and thawed on ice. Once thawed up to 2 µl ligated DNA was added to 50 µl competent cells. Electroporations were made in prechilled 2mm cuvettes using a Gene Pulser (Biorad) set at 2500 V, 25 µF, 200 Ω, 5msec pulse. One ml SOC medium (2 % tryptone, 0.5 % yeast extract,

10 mM NaCl, 10 mM MgSO₄, 10mM MgCl₂) was added to the cuvette immediately after the pulse. The bacteria were transferred to a 15 ml centrifuge tube and incubated for one hour at 37°C whilst shaking to allow the expression of the antibiotic resistance conferred by the plasmid. Bacteria were plated on selective agar plates and incubated at 30°C overnight.

2.1.13 Colony lift

Plates with 50-200 colonies (where discrete colonies could still be easily identified) were used for colony lifts. Fresh LB agar plates containing 100 µg/ml ampicillin and 10µM isopropyl thio-galactoside (IPTG, BDH) were used. Round hybond filter paper (HybondTM-N+, Amersham International) was marked with a ballpoint pen and placed on the bacteria with blunt forceps. The agar plate was marked for orientation. The filter paper was removed, inverted and placed colony side up onto the IPTG plate, easing out air bubbles between the filter paper and plate. The IPTG plate was incubated at 30°C overnight. The original plate was left on the bench overnight to allow regrowth of bacteria before storage at 4°C. The following morning colonies were visible on the transferred filter which was then removed for colony lysis and staining. Solutions used in this procedure were *SDS solution* (10% sodium dodecyl sulphate), *denaturing solution* (0.5M NaOH; 1.5M NaCl), *neutralisation solution* (1.5M NaCl; 0.5M Tris-HCl pH 7.4), *2x SSC solution* (made as 20x : 87.65g NaCl; 50.25g trisodium citrate dehydrate in 500ml dH₂O) and blocking buffer (PBS, 3% skimmed milk, 0.05% NaN₃).

Five pieces of WhatmanTM 3MM filter paper were labelled 1-5 and wetted with the following solutions. The Hybond -N+ filter was then placed colony side up onto each Whatman 3MM filter for the specified times

1)	<i>SDS Solution</i>	10 min
2)	<i>Denaturing Solution</i>	5 min
3)	<i>Neutralisation Solution</i>	5 min
4)	<i>Neutralisation Solution</i>	5 min
5)	<i>2x SSC Solution</i>	15 min

The nitrocellulose filter was then placed colony side up in *blocking buffer* and shaken gently at room temperature for 4 hours. The colonies were then gently rubbed off under running water and the filter returned to fresh blocking buffer and left shaking overnight. Thereafter the colony lift filter was processed as filters after Western blotting. The development was terminated with tap water once sufficient darkening of the background allowed visualisation of both negative (white) and charcoal (positive) colonies.

2.2 PROTEIN PURIFICATION TECHNIQUES

Following cloning of the fusion molecules and confirmation of the DNA sequence the protein was purified from *E. coli*. The steps for protein purification are described.

2.2.1 Storage of Bacterial Clones

All bacterial clones were stored at -80°C as glycerol stocks. Five hundred μl of bacterial overnight culture was mixed with 500 μl of sterile glycerol, vortexed briefly to mix and then stored in a CryovialTM (Nalgene) at -80°C .

2.2.2 Bacterial Overnight Cultures

Overnight cultures of bacterial clones were prepared by picking bacterial colonies from an agar plate or by stab-sampling a glycerol stock of the clone of interest. The bacteria were used to inoculate 1.5 ml of LB (Luria Broth, Life Technologies) containing 2% D-glucose and, where appropriate, 100 μg ampicillin (Boeringer Mannheim) per ml (LB_{GA}). This suspension was then shaken at 250rpm, 30°C overnight.

2.2.3 Preparation of Bacterial Supernatants

All scFvs were isolated as secreted proteins from bacterial supernatants. Overnight cultures were diluted 1/100 in the morning in LB_{GA} and shaken at 250rpm for 6 hours at 30°C to an OD₆₀₀ of approximately 1.5. For small-scale inductions a volume of 1.5ml was used and for large-scale inductions a 500ml culture was produced from a 10ml overnight culture. Induction of protein production was carried out using isopropyl-thio-galactoside (IPTG) in the absence of glucose. The bacteria were

pelleted (3000rpm, 10min, RT) and the supernatant discarded. For small-scale inductions, the bacteria were resuspended in LB containing 100µg ampicillin per ml and 500µM IPTG (LB_{AI500}). For large-scale inductions, the bacteria were resuspended in 1 litre of LB containing 100µg ampicillin per ml and 100µM IPTG (LB_{AI100}). A 2.5 litre baffled flask containing 1 litre of broth was used. Cultures were then shaken overnight (for 16hours) at 30°C and 250rpm.

Small-scale induction supernatants were analysed without purification after pelleting the bacteria at 13000rpm for 5min in a benchtop centrifuge.

For large inductions, 0.05% sodium azide was added as a bacteriostatic agent and 200µM PMSF (phenyl-methyl-sulphonyl-fluoride) to reduce proteolysis. The culture was then centrifuged at 18500 g at 4°C for 1 hour in a cold centrifuge (Beckman, JA-10.500 rotor). The supernatant was passed through a 0.2µm filter (Dow Corning) and subsequently concentrated to approximately 50-100ml using a tangential flow filtration device (Fig. 2.1) with a 10 kDa filtration threshold (Ultrasette™, Filtron). Bacterial supernatant is pumped through the Ultrasette™ filter. Water and proteins below 10kDa are forced to flow across the membrane due to backpressure generated by the clamp. The scFv is too large to pass through the pores in the membrane and thus is retained. The sample was dialysed, overnight at 4°C, against 2 litres PBS pH 7.5 and then purified the following morning.

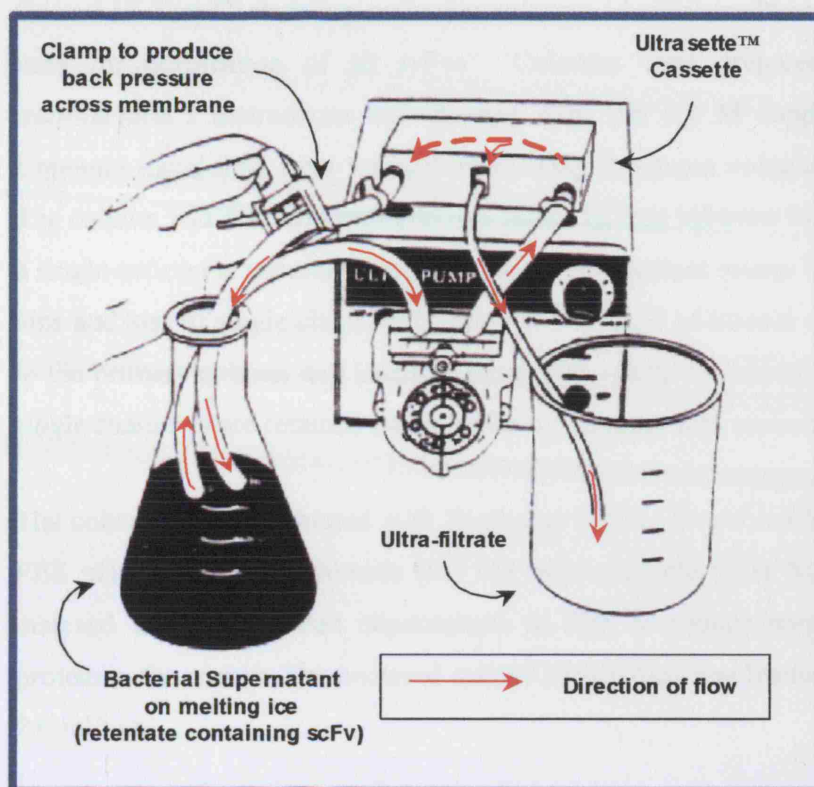


Figure 2.1: Bacterial supernatant is pumped through the Ultra sette™ filter at approximately 800ml/min. Water and proteins smaller than 10kDa are forced across the membrane due to backpressure (5ml/min) adjusted by the clamp. The scFv (28 kDa) does not pass through the pores in the membrane and is retained. Once concentrated to approximately 100 ml the supernatant is dialysed against 2 litres PBS pH 7.5 overnight at 4°C and then purified the following morning (see IMAC section).

2.2.4 Purification of scFvs by Immobilised Metal-ion Affinity Chromatography (IMAC)

A poly-histidine oligopeptide (6 histidine residues) has a high affinity for specified metal ions including Zn^{2+} , Cu^{2+} and Ni^{2+} . Proteins incorporating a poly-histidine sequence can therefore be affinity purified by selective binding to metal ions immobilised on a solid medium (IMAC). The development of HiTrap gels (Amersham Pharmacia Biotech) that chelate metal ions has allowed the purification of numerous proteins including scFvs on a column system based on this principle (Casey *et al.*, 1995). The Biologic LP platform (Biorad) was used for all purifications (Figure 2.2).

One ml Hi-Trap[™] chelating sepharose columns (Amersham Pharmacia Biotech) were used for purification of all scFvs. Columns were prepared according to the manufacturer's instructions and charged with 1ml 0.1 M copper sulphate (BDH). Unbound metal ions were washed away using 5 column volumes of distilled water. The column was then connected to two further HiTrap columns in tandem. The use of a single column is unsuitable as the bacterial supernatant causes leaching of the metal ions and loss of single chain Fv protein. However, if additional columns are attached to the primary column any leaching metal ions rebind to subsequent columns. Thus, single chain Fvs are retained on the series of columns until elution.

The column was equilibrated with 20ml start buffer (10mM imidazole, 0.5M NaCl in PBS pH7.4). Sodium chloride (0.5 M) and imidazole (0.01 M) were added to the dialysed and concentrated supernatants in PBS to reduce non-specific binding of protein to the matrix. The bacterial culture supernatant was loaded onto the column at 2.0 ml/min.

The column was washed with a further 20ml start buffer to remove any unbound protein. Competitive elution was then carried out with a stepwise gradient of imidazole (Sigma Aldrich) in PBS at 2 ml/min. Twenty ml of 30 mM, 50 mM and then 200 mM imidazole in PBS plus 0.5 M NaCl, pH 7.5 were used. All samples collected were concentrated by ultrafiltration using Vivaspin concentrators (Vivascience).

To regenerate the column, the metal ions and any remaining protein on the column were then eluted using 20ml 0.1M EDTA followed by 60 ml distilled H₂O at 2 ml/min. The column was washed with 20 ml of 20% ethanol and stored at 4°C, ready for re-use.

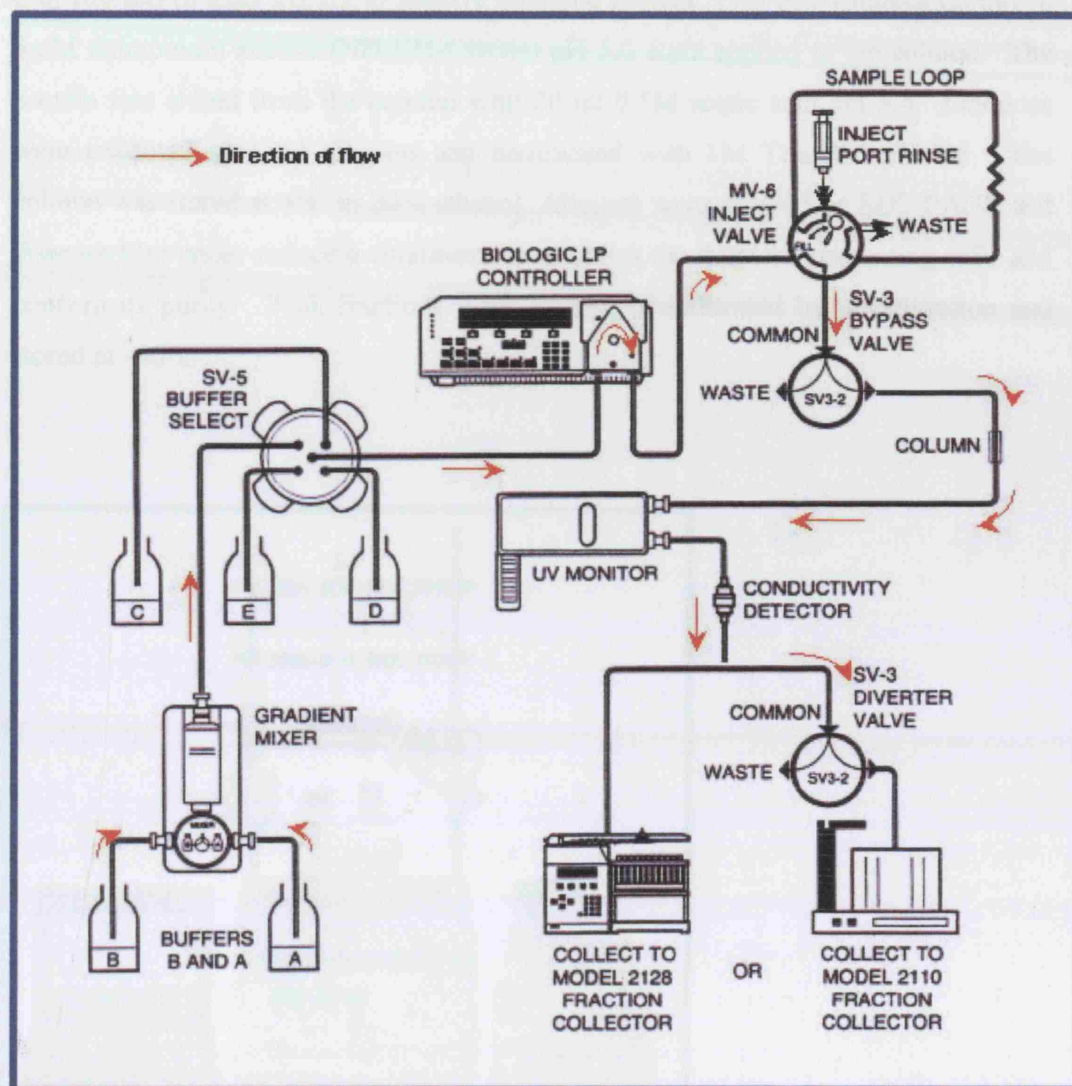


Figure 2.2: Setup of the Biologic LP Platform for use with HiTrap columns. The flow direction is indicated.

2.2.5 Purification of ZZ Fusion Proteins by IgG Sepharose Chromatography

An IgG Sepharose[®] 6 Fast Flow column (Amersham Pharmacia Biotech) was equilibrated with two cycles of 30ml 0.5M acetic acid pH3.4, followed by 30 ml TST buffer (50mM Tris acetate buffer pH 7.6, 150mM NaCl and 0.05% Tween 20). The column setup is shown in Figure 2.3.

The concentrated and filtered scFv dialysed against PBS was loaded onto the column at 0.5 ml/min. An aliquot of the supernatant and the run through were collected and stored at 4°C for further tests. The column was washed with 5-10 column volumes (50–100 ml) of PBS pH 7.2 to remove unbound protein. Two bed volumes (20ml) of 5mM ammonium acetate ($\text{NH}_4\text{CH}_3\text{COOH}$) pH 5.0 were applied to the column. The protein was eluted from the column with 20 ml 0.5M acetic acid pH 3.4. Fractions were collected in 1 ml aliquots and neutralised with 1M Tris/HCl pH 8.8. The column was stored at 4°C in 20% ethanol. Aliquots were assayed by SDS-PAGE and Western blot under reducing conditions to establish the fractions containing scFv and confirm its purity. Peak fractions were pooled, concentrated by ultrafiltration and stored at -80°C.

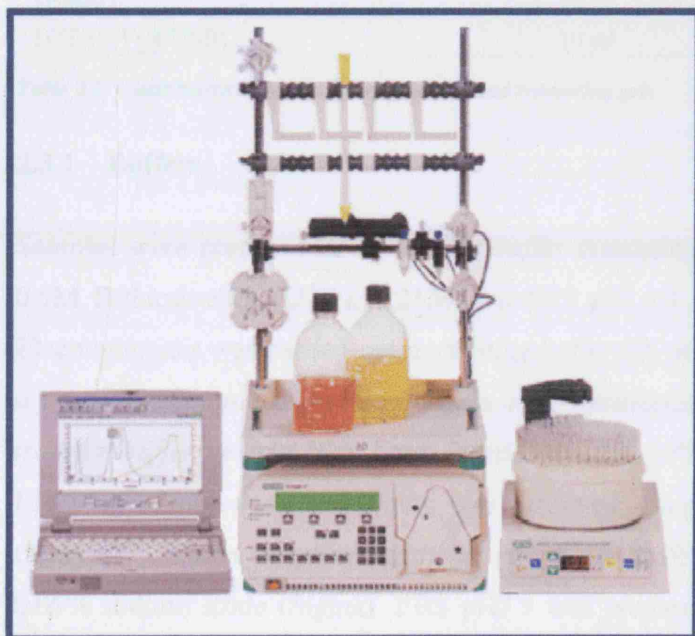


Figure 2.3: Typical set up for the purification of ZZ fusion proteins.

2.3 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE)

Gel Casting

Protein electrophoresis was carried out using Mini-Protean™ gel electrophoresis equipment (Bio-Rad). The samples were separated using a 4% SDS stacking gel and a 15% separating gel. The gels were prepared as shown in Table 2.1.

	Stacking gel	Separating gel
	4%	15%
Acrylamide/Bis (30%)	1.3 ml	5.0 ml
Distilled H ₂ O	6.1 ml	2.4 ml
3.0 M Tris-HCl, pH 8.8	0.0 ml	2.5 ml
0.5 M Tris-HCl, pH 6.8	2.5 ml	0.0 ml
10% SDS	100 µl	100 µl
Ammonium persulphate (100 mg/ml)	50 µl	50 µl
TEMED	10 µl	10 µl
TOTAL VOLUME	10 ml	10 ml

Table 2.1: Constituents used to prepare stacking and separating gels.

2.3.1 Buffers

Samples were prepared in 5x *sample buffer* consisting of 50% glycerol, 10% SDS, 0.5M Dithiothreitol (DTT), 0.25M Tris-HCl pH 6.8 and 0.5% bromophenol blue. Electrophoresis was carried out in *running buffer* (25 mM Tris-base, 192 mM glycine, 0.1% SDS). Separated protein samples were transferred to nitrocellulose paper using *transfer buffer* (48mM Tris base, 40mM glycine, 20% methanol and 0.05% SDS). Blocking of non-specific binding was achieved using 3% skimmed milk solution (Marvel™, Tesco Supermarkets) in phosphate-buffered saline (PBS) containing 0.05% sodium azide (Sigma). PBS pH7.5 was prepared as 26.5 mM KCl, 147 mM KH₂PO₄, 80.6 mM NaCl and 8.06 mM Na₂HPO₄ in distilled water. All antibodies used for staining the filters were diluted in *incubation buffer* containing 0.1% Bovine Serum Albumin (BSA) and 0.05% Tween-20 in PBS. *Wash buffer* for Western blots consisted of 0.05% Tween-20 in PBS.

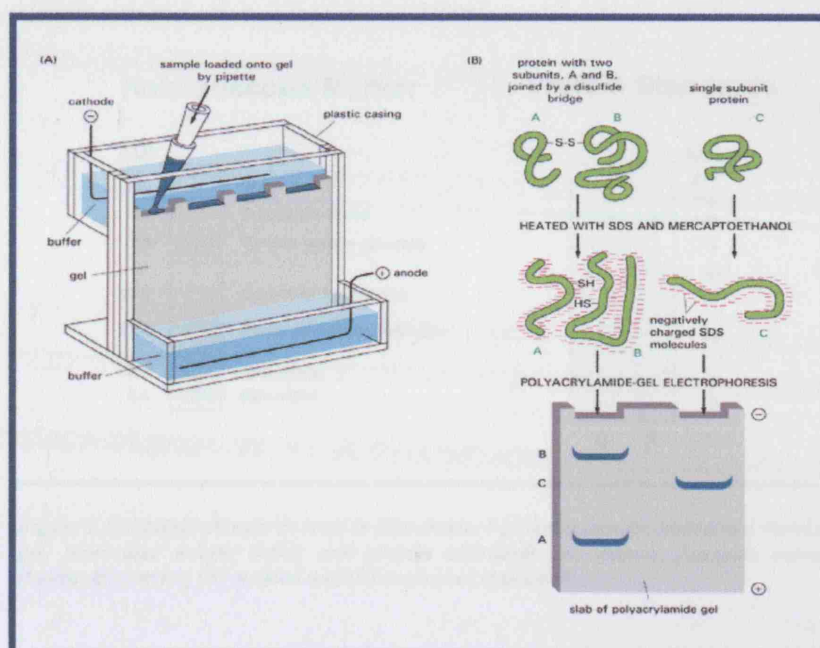


Figure 2.4: Principle of SDS-PAGE. The proteins are heated with SDS and mercaptoethanol and electrophoresed. The sample preparation process breaks up the disulphide bonds enabling the protein subunits to be seen by Coomassie brilliant blue staining and specific protein subunits to be detected by Western blot analysis.

2.3.2 SDS-PAGE

The principle of SDS-PAGE is illustrated in Figure 2.4. SDS PAGE gels were poured following manufacturer's instructions. Twenty μl samples with 5 μl 5 x reducing buffer were denatured at 95°C for 3 minutes. Known amounts of ovalbumin (1 μg , 3 μg and 5 μg) were also loaded to allow quantification of scFv. Once the electrophoresis (1 hour at 200V) was completed, the gel was stained with Coomassie brilliant blue. Coomassie brilliant blue stain is a protein stain prepared by dissolving 1g Coomassie dye in 200 ml destainer (20ml glacial acetic acid, 90 ml methanol and 90 ml water). The gel was stained for 30 - 60 minutes and then destained in Coomassie destainer for approximately 24 hours before drying on Whatman 3MM paper. This allowed quantification of protein and determination of protein size and purity. Protein fragment size was estimated in comparison to Kaleidoscope™ Prestained Protein Markers (Bio-Rad) or Low Range Molecular Weight Standards (Biorad) (see Figure 2.5).

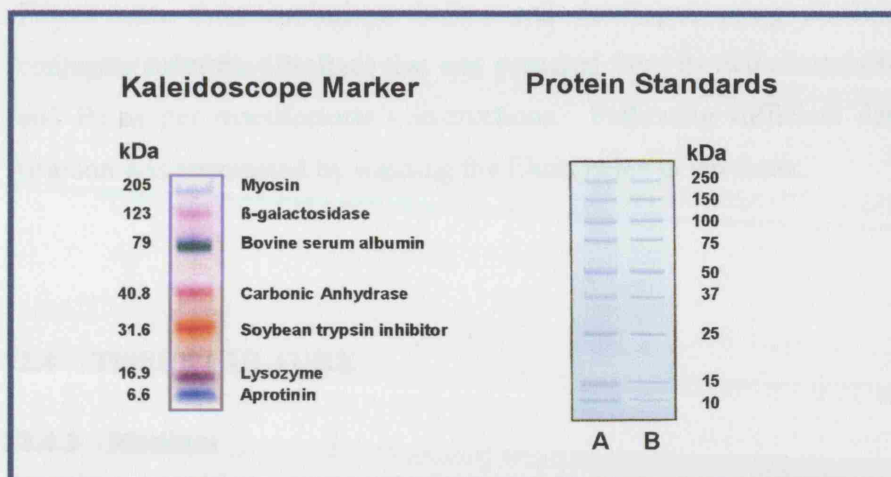


Figure 2.5: Protein standards used in this thesis. 5 μ l Kaleidoscope prestained standards run on a 4-20% ready gel. Molecular weight (kDa) and protein standards are shown alongside corresponding bands. Protein standards showing (A) stained and (B) unstained standards.

2.3.3 Western Blot Analysis

Western blot analysis of proteins was carried out according to standard methods. Protein samples and gels were prepared as for SDS-PAGE analysis and electrophoresis was carried out using the Mini-Protean™ III system (BioRad) at 200 volts for 60 minutes. Transfer of the separated proteins onto Hybond ECL membrane (Hybond™ Amersham International) was achieved using a semi-dry electroblotting unit (Z34,050-2, Sigma-Aldrich). Six pieces of Whatman™ 3MM filter paper measuring 7 x 9 cm were soaked in *transfer buffer*. Three pieces of pre-soaked filter paper were placed on the bottom (anode) plate of the blotting unit, followed by the nitrocellulose membrane. The gel was placed onto the nitrocellulose membrane and then a further three pieces of 3MM filter paper were placed on top of this. A roller was used to gently ease out any air bubbles from between the paper sheets. The top (cathode) electrode plate was placed onto the stack, closing the blotting unit. Protein was transferred at 100 V and 200 mA for 1 hour.

After transfer the Hybond ECL nitrocellulose membrane (AP Biotech) was incubated in *blocking buffer* at room temperature overnight. The following morning the membrane was washed three times with wash buffer (PBS with 0.05% Tween-20) and the proteins on the filter were detected using various antibodies coupled to alkaline phosphatase (further detection details are described in the appropriate chapters).

Filters were then washed as before and developed using alkaline phosphatase conjugate substrate (BioRad) that was prepared from its two components (reagents A and B) as per manufacturer's instructions. Following sufficient development the reaction was terminated by washing the filters twice in tap water.

2.4 TISSUE CULTURE

2.4.1 Medium

All cell lines were maintained in RPMI medium (Life Technologies) supplemented with 2mM L-glutamine (Gibco) and 10% foetal calf serum (FCS, Gibco). To retard bacterial growth, penicillin and streptomycin were added to the medium. This is hereafter referred to as "*culture medium*".

Tissue culture procedures were carried out in a Class II Hood (Greiner). Medium, PBS and VerseneTM (Gibco) were pre-warmed to 37°C before use. All cell cultures were incubated at 37°C in a tissue culture incubator (Jencons) in an air atmosphere supplemented with 5% CO₂.

2.4.2 Freezing Cell Lines

Following sufficient growth of cells they were frozen down as stocks for future use. At confluence, the *culture medium* was aspirated and the cells released by incubation with Versene 1:5000 (Life Technologies) at 37°C for ~ 10min. An equal volume of *medium* was added and the cells pelleted at 1000rpm for 5 min. The cell pellet was reuspended drop-wise with 5ml freezing medium (1ml dimethyl sulphoxide (DMSO) (Sigma Aldrich), 9ml FCS). One ml aliquots were added to CryovialsTM (Nalgene). The cryovials were wrapped in 4-5 layers tissue and stored at -80°C for 1 week. They were then removed from the tissue and transferred to liquid nitrogen for long term storage. Cells were stored at -80°C before transferring to liquid nitrogen as this is a more gentle procedure for the cells enabling maximal recovery of cells when taken back into culture.

2.4.3 Taking Cell Lines Into Culture

Frozen stocks (10^6 cells) in 1ml dimethyl sulphoxide (DMSO) (Sigma Aldrich) / FCS (1:9) from liquid nitrogen storage were thawed rapidly in a 37°C water bath and resuspended by drop-wise addition of 10ml *culture medium*. The cells were pelleted at 1000rpm for 5min in a benchtop centrifuge. The cells were resuspended in 5ml of *culture medium* and seeded into a T25 culture flask (Greiner).

2.4.4 Maintenance Of The A375-M Cell Line

The human melanoma cell line A375M (Giard *et al.*, 1973) was obtained from the European Catalogue of Human and Animal Cell Culture (ECACC) and grown using standard tissue culture techniques.

At confluence, the *culture medium* was aspirated and the cells released by incubation in 1:5000 Versene (Life Technologies) for ~ 10min at 37°C. An equal volume of *medium* was added and the cells were pelleted at 1000rpm for 5 min. When passaging cells, the pellet was resuspended in a small volume of *culture medium* prior to seeding T75 flasks (Greiner) containing 25ml of *culture medium*. Typically, cells were split 1:5. When larger cell numbers were required, the cells were passaged into T175 flasks (Greiner) containing 50ml *culture medium*.

2.4.5 Maintenance Of The SK28 Cell Line

The human melanoma cell line SK28 (Carey *et al.*, 1976) was obtained from the European Catalogue of Human and Animal Cell Culture (ECACC) and grown using standard tissue culture techniques. SK28 is a cell line derived from a 51 years old male melanoma patient and expresses the high molecular weight melanoma-associated antigen (Noronha *et al.*, 1998).

At confluence, the *culture medium* was aspirated off and the cells were released by incubation with 1ml trypsin (0.5%) diluted in 9ml 1:5000 Versene (Life Technologies) for ~ 5min at room temperature. An equal amount of *culture medium* was added and the cells were pelleted at 1000rpm for 5 min. The pellet was resuspended in 5ml *culture medium*. Typically, cells were seeded 1:4 in T75 flasks

(Greiner) containing 25ml of *culture medium*. Flasks typically reached confluence 3 days after seeding.

2.4.6 Maintenance of the U937 Cell Line

Cells were brought into culture as described in section 2.4.3. At confluence the cells were pelleted at 1000rpm for 5 minutes. The pellet was resuspended in 8ml *culture medium*. The cell suspension was used to seed T175 flasks (Greiner). Cells were split 1:8. Flasks typically reached confluence after 7-10 days of seeding. Cells maintained for 3-5 passages were suitable for ADCC assays.

2.4.7 Preparation of Cell ELISA Plates

Purified HMW-MAA is not available. *In vitro* analysis of scFv-antigen interaction was therefore carried out using cell ELISA plates seeded with A375M melanoma cells.

Cells from a single T75 flask at confluence (approximately 8×10^6 cells) were harvested and resuspended in 50ml *culture medium*. This suspension was used to seed ten 96-well flat-bottom tissue culture plates (Falcon) aliquoting 50µl per well ($\sim 8 \times 10^3$ cells). The plates were incubated for 3-4 days in a food box coated with damp paper towels and placed inside the tissue culture incubator, until slightly under-confluent. The *culture medium* was then aspirated and the plates dried overnight in a 37°C incubator before storage at 4°C for several weeks.

2.5 ANIMAL MODEL

2.5.1 Tumour Cell Preparation

A375-M tumour cells were used for all animal experiments. Cells were cultured as described in Section 2.4.4 but in the absence of antibiotic. Cells were harvested from confluent flasks under sterile conditions less than 1 hour prior to inoculation of mice.

2.5.2 Implantation of Human Tumour Xenografts in Mice

Female immuno-deficient Balb/c nu mice were used for all experiments as a tumour xenograft model. Mice were obtained from existing breeding stock at the animal house (Gray Cancer Institute, Mount Vernon Hospital). Standard aseptic techniques were used in all procedures involving immuno-compromised mice according to the animal house protocols. The mice were housed in MB1 plastic cages (North Kent Plastics) holding a maximum of 20 mice and fed on a diet of standard expanded pellets (B & K Universal). Mice were implanted with tumour cells when they reached between 7 to 10 weeks of age.

Hands and forearms were scrubbed with antiseptic agents before touching the animals. All clothing except underwear was removed before entering the animal house and surgical hats, surgical masks, dedicated (clean) overalls, latex gloves and operating boots were worn at all times.

A clean operative field was prepared by laying tissue paper onto the bench-top and spraying this with 70% ethanol. The cell suspension was drawn up into a 1 ml syringe (NHS supplies) and a 23 G x 1" (Terumo™ "blue-hub") needle was fixed on the end of the syringe.

Ten million cells were used (unless otherwise specified) for inoculation of each animal. Cells were resuspended in sterile PBS to a volume of 100µl per 10 million cells and injected subcutaneously into the flank of a Balb/c nu mouse under Enflourane™ anaesthesia (methoxyfluorane, Janssen Pharmaceutical). A small subcutaneous bleb was raised by the tumour bolus at the site of inoculation on the right flank. The animals were then returned to their cages after recovery from anaesthesia and observed on a regular basis.

2.5.3 Immunisation of Mice

CBA mice were used for all immunisations. Mice were obtained from existing breeding stock in the animal house (Gray Laboratory, Mount Vernon Hospital). Standard aseptic techniques were used for all procedures according to the animal house protocols. The mice were housed in MB1 plastic cages (North Kent Plastics)

holding a maximum of 20 mice and fed on a diet of standard expanded pellets (B & K Universal). Mice were immunised when they reached between 6 to 10 weeks of age.

A clean operative field was established as in section 2.5.2 above. The protein/adjuvant suspension was drawn up into a 1 ml syringe (NHS supplies) and a 23 G x 1" (Terumo™ "blue-hub") needle was fixed onto the end of the syringe. Mice were immunised intraperitoneally with a suspension consisting of 25µg protein in 100µl PBS and 100µl Complete Freund's Adjuvant (Sigma). Repeated boosters were given every 3 weeks consisting of 25µg protein in 100µl PBS and 100µl Incomplete Freund's Adjuvant until the completion of the experiment. Ten days post immunisation blood samples were taken from the tail vein of immunised mice. Tail vein vasodilation was induced by gently heating using an infrared lamp. The animals were then returned to their cages after recovery from anaesthesia and observed on a regular basis.

Chapter 3

***In Vitro* Characterisation of R3ZZ**

3 IN VITRO CHARACTERISATION OF R3ZZ

3.1 INTRODUCTION

3.1.1 Current therapies

The current treatment options for melanoma patients are limited. The main form of treatment for localised melanoma is surgical excision with margins proportional to the microstage of the primary lesion (Kelly *et al.*, 1984) [Chapter 1 Section 1.2].

If melanoma is detected at stage I the five year survival rate may vary from 84 to 95% depending on the depth of the invasion. Stages II and III show typical 5 year survival rates between 60 to 70%. If the melanoma is diagnosed at stage IV the 5 year survival incidence is reduced to 16% (Slingluff *et al.*, 1992). If melanoma is not detected at an early stage, there is also a high risk of recurrence in patients.

In patients with metastatic disease, chemotherapy, biologic therapy (e.g. interleukin 2 [IL-2], tumour infiltrating lymphocytes, lymphokine activated killer cells) and combination biochemotherapy have yielded low response rates of 20-30% (Rosenberg *et al.*, 1994; Rosenberg *et al.*, 1997). These are discussed in greater depth in Chapter 1 Sections 1.2.2 – 1.2.3. Adjuvant treatment of melanoma with chemotherapy or immunotherapy after radical surgery for regional lymph node metastases has not, until now, shown any substantial benefit (Cascinelli *et al.*, 2003).

3.1.2 Immune Response in Melanoma

A study demonstrating inhibition of tumour growth by the administration of *Corynebacterium parvum* (Halpern *et al.* 1966) and the success of the BCG (*Bacillus Calmette-Guerin*) vaccine in eradicating some tumours (Key and Hanna. 1981a; 1981b) led to a revival of studies utilising the immune response for the therapy of cancer. The development of novel approaches using active specific immunotherapy with melanoma vaccines have been prompted by clinical observations suggesting an important role of the immune system in melanoma (Morton *et al.*, 1970). Efforts to understand the immune response to melanoma have made it the most intensively studied cancer in the field of tumour immunology (Polsky *et al.*, 2001). However,

melanoma also induces immune tolerance. In addition, it exhibits genomic instability. Therefore, variant clones are generated throughout the course of the disease leading to immune escape (Runger *et al.*, 1994). Chapter 1 Sections 1.1.10.5 – 1.1.13 discuss immune tolerance and escape in melanoma in detail.

3.1.3 Tumour Associated Antigens

A number of studies have reported an immune response to melanoma (Geertsen *et al.*, 1999; Mukherji *et al.*, 1995). These are discussed in detail in Chapter 1 Section 1.8. Both antibody and T cell immune recognition of melanoma have been shown in humans. Antibodies against autologous melanoma have been detected in the serum of patients, and three classes of antigens recognised by these antibodies have been described. These antigen classes are tumour-associated cancer testis-specific antigens, melanocyte differentiation antigens and mutated or aberrantly expressed self-antigens and are discussed in detail in Chapter 1 Section 1.1.10.

Effective immunity against cancer requires the specific recognition and elimination of malignant cells expressing targeted antigens (Houghton, 1994). The identification and molecular characterisation of human tumour associated antigens has identified well-defined targets for the active specific immunotherapy in patients with malignant tumours (Greten *et al.*, 1999). Since the 1980s identification of tumour associated antigens (TAAs) has prompted the development of different strategies for anti-tumour vaccination aimed at inducing specific recognition of TAAs in order to elicit a persistent immune memory that may eliminate residual tumour cells and protect patients from relapse post surgery (Bocchia *et al.*, 2000).

3.1.4 Immunotherapy

Disease and drug related immunodeficiencies are major research areas. Many of the deadly diseases like cancer, AIDS and the drugs used in their treatment cause severe immunodeficiencies (Cohen *et al.*, 1993). As a result immunodeficient patients often die from secondary infections rather than the primary disease itself. Thus immunomodulators such as interleukins, tumour necrosis factor and interferon γ are under investigation as attempts to overcome immunodeficiency (Tas *et al.*, 1999). Studies on the administration of interleukin-2 to patients with metastatic melanoma or

kidney cancer have shown that immunological manipulations can mediate the durable regression of metastatic cancer (Rosenberg, 2001). However, non-specific immunomodulators can also have serious side effects.

For many years the field of immunotherapy of malignant diseases was divided into two distinct, and at times antagonistic camps with one emphasising the importance of the cellular and the other the humoral effector arm of the immune system for the destruction of tumour cells. In the following years appreciation of the complementary nature of T-cell and antibody based immunotherapy stimulated interest in developing approaches that combine their advantages and minimise their limitations (Abken *et al.*, 1998). Furthermore, the realisation that tumour cells utilise multiple mechanisms to escape from immune recognition and destruction has stimulated interest in developing and applying immunotherapeutic strategies that target both humoral and cellular immunity to malignant cells (Wang *et al.*, 2000). Examples of such strategies are described in Chapter 1 Sections 1.2.5 – 1.2.7.

3.1.5 Antibody dependent cell mediated cytotoxicity

Antibody dependent cellular cytotoxicity (ADCC) is considered to be one of the most important defence mechanisms in response to neoplasia and viral infections (Koch *et al.*, 1989). Tumour destruction results from three major mechanisms: Firstly, destruction of the tumour by necrosis mediated by polymorphonuclear leukocytes, secondly, indirect inhibition of angiogenesis by secondary interferon γ (IFN- γ), tumour necrosis factor α (TNF- α) and chemokines; and finally activation of leukocyte subsets capable of producing proinflammatory cytokines and generating CTLs and antitumour Abs (Cavallo *et al.*, 1999).

3.1.5.1 Role of CTLs

Studies on T-cell activity in cancer patients with primary cancer lesions showed up to 70% positive reactions measured as proliferation or significant lysis (Fossati *et al.*, 1984). Studies in animal models indicate that a minimal level of expression of major histocompatibility complex (MHC) class I by the tumour is necessary to trigger a T-cell response through the T-cell receptor (TCR) complex. *In vitro* studies have revealed a similar situation in human neoplasms. The use of tetrameric soluble

peptide–MHC class I complexes to identify tumour-specific CD8⁺ T cells have allowed rapid and accurate analysis of human CD8⁺ T-cell responses in cancer patients (Romero *et al.*, 1998).

Tumour-specific MHC-restricted CTLs are cytotoxic effector cells that lyse neoplastic cells but not normal autologous cells. Such CTLs, generally CD3⁺CD8⁺ cells, interact through their TCR/CD3 complex with antigenic peptidees bound to MHC class I molecules on the surface of tumour cells (Slovin *et al.*, 1986). In addition, adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and its ligand on lymphocytes, leukocyte function-associated antigen 1 (LFA-1), promote costimulatory signals for TCR-mediated activation of resting T cells (VanSeventer *et al.*, 1990) and play an essential role in modulating interactions between T cells and tumour cell targets.

Cytotoxic effector cells mediate tumour destruction by releasing perforins (pore-forming proteins), serine esterases and IFN- γ and/or TNF- α (Berke, 1989). This mechanism is illustrated in Figure 3.1. The T cell response is therefore highly relevant in the clinical control of cancer development and progression. Studies with animal tumours using tumour-specific T cells in combination with interleukin 2 (IL-2) transferred to tumour-bearing animals resulted in eradication of the malignancy (Rodolfo *et al.*, 1990).

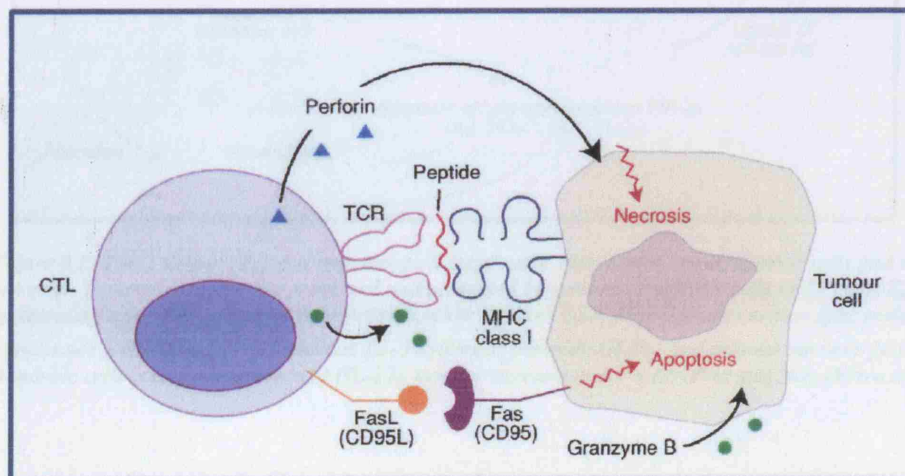


Figure 3.1. The cytotoxic T lymphocyte (CTL) response to tumour cells. CTLs interact with tumour cells by binding T-cell receptor (TCR) to the major histocompatibility complex (MHC) class I molecules expressed on the tumour cell surface. The interaction between adhesion molecules and Fas (CD95) with Fas ligand (CD95L) are shown (Mitra *et al.*, 2003).

3.1.5.2 Role of T helper (T_H) cells

$CD4^+$ T_H cells play an important role in the amplification and regulation of the cellular immune response (Figure 3.2). Antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs), engulf tumour cells. Tumour cell antigens are processed and presented to T_H cells via MHC class II molecules, which interact with the TCR on T_H cells (Wang, 2001). T_H cells respond by secreting cytokines, which in turn activate other immune cells. T_H cells are divided into two types according to their cytokine profile (Mosmann and Sad 1996). T_H1 cells are characterised by the production of $IFN-\gamma$, IL-2, and IL-12 whereas T_H2 cells produce IL-4, IL-5 and IL-10. T_H1 cells stimulate a CTL response and T_H2 cells a humoral immune response.

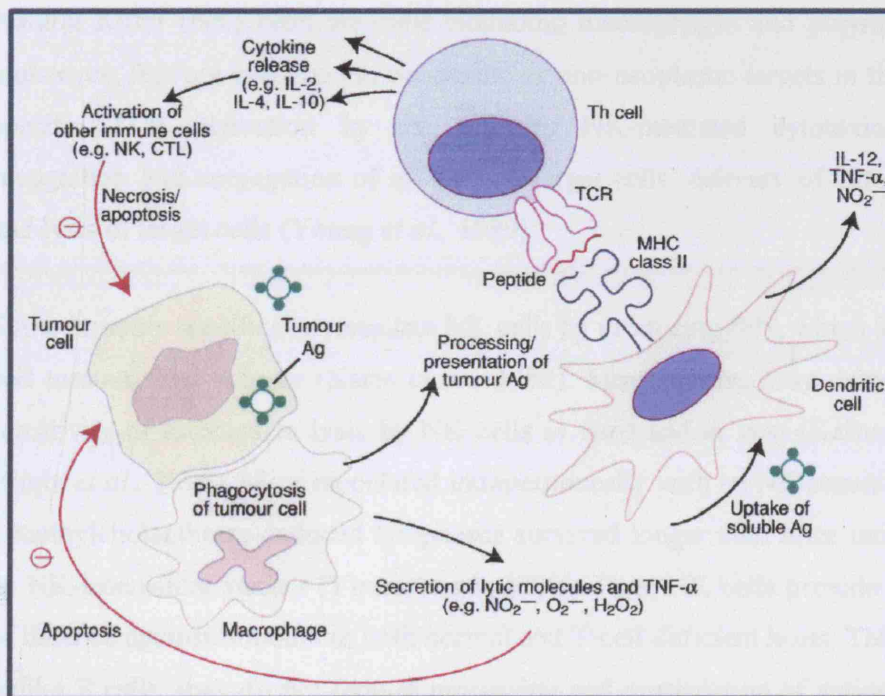


Figure 3.2. The T helper (T_H) cell response to tumour cells. Phagocytes engulf tumour cells and release tumour antigens. Tumour antigens are processed and presented by antigen presenting cells to T_H cells. T_H cells respond by secreting cytokines, which in turn activate other immune cells. Macrophages secrete lytic molecules, such as nitric oxide (NO_2^-), superoxide anions (O_2^-) hydrogen peroxide (H_2O_2) and tumour necrosis factor α (TNF- α). Dendritic cells secrete interleukin12 (IL-12), tumour necrosis factor α (TNF- α) and NO_2^- (Mitra et al., 2003).

The discrimination of T_H cells into T_H3 (Inobe *et al.*, 1998) or T regulatory 1 (T_R1)-type cells (Groux *et al.*, 1997) is characterised by the production of IL-10 and other inhibitory cytokines such as transforming growth factor β (TGF- β). T_H0 cells produce

a wide spectrum of cytokines. Different subsets of T_H cells play an important role in regulatory and controlling the initiation and downregulation of the immune response. Research has focused mainly on antitumour CTL responses, rather than T_H-cell responses because a majority of tumours express MHC class I but lack MHC class II (Melief *et al.*, 2000).

However, it has become evident that, for efficient and long lasting immunity against tumours, activation of tumour-specific T_H cells is essential. For example, CD4⁺ T cells are crucial for elimination of foetal bovine lung (FBL) erythroleukaemia (Greenberg, 1991).

3.1.5.3 Role of Natural Killer cells

Natural Killer (NK) cells are cells excluding macrophages and polymorphonuclear leukocytes that are cytotoxic to neoplastic or non-neoplastic targets in the absence of specific TCR activation by the antigen. NK-mediated cytotoxicity involves recognition and conjugation of effector to target cells, delivery of cytotoxic signals and lysis of target cells (Young *et al.*, 1989).

Some tumours specifically stimulate NK cells by producing IFN, which activates NK-cell tumouricidal activity (Karre *et al.*, 1989). Many studies have demonstrated the sensitivity of tumours to lysis by NK cells *in vitro* and *in vivo* (Karre *et al.*, 1989; Bright *et al.*, 1995). Mice inoculated intraperitoneally with an NK-sensitive variant of a methylcholanthrene-induced lymphoma survived longer than mice inoculated with an NK-insensitive variant (Young *et al.*, 1989). Thus, NK cells provide the first line of defence against tumours in both normal and T-cell-deficient hosts. This is because, unlike T cells, they do not require processing and presentation of antigenic peptides by MHC molecules.

Therefore, NK cells target tumour cells that escape T cell mediated kill because they do not express the appropriate MHC molecules (Figure 3.3). Patients with low NK cell activity have lower resistance to infection and are at increased risk of cancer metastasis formation (Trinchieri, 1989). If NK cells fail to produce cytolytic factors following contact with target cells they lose their kill activity. NK cells that have been inactivated by target cells are characterised by downregulation and/or shedding of the FcγRIII (CD16) receptor. These observations suggest that CD16 may play an active role in target-cell induced NK cell anergy and apoptosis.

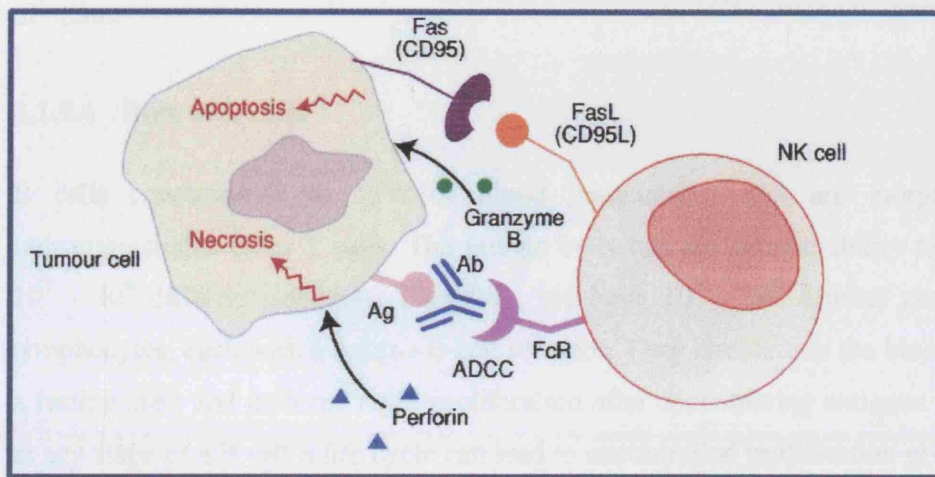


Figure 3.3. The natural killer (NK) cell response to tumour cells. NK cells kill tumour cells by several mechanisms that involve: antibody (Ab)-dependent cellular cytotoxicity (ADCC), in which the Fc portion of an Ab bound to antigen (Ag) on the tumour cell surface binds to the Fc receptor (FcR) on the NK cell; Fas (CD95)–Fas ligand (CD95L) interaction; and release of perforin and granzyme B molecules, which cause apoptosis/necrosis of the tumour cell (Mitra *et al.*, 2003).

Target cell-induced anergy in NK cells has been demonstrated by co-culture with fixed AK-5 tumour cells (a rat histiocytic tumour cell line) (Das *et al.*, 2000). In addition, TNF- α secretion and Fas ligand (CD95L) upregulation on NK cells after coculture plays an important role in inducing NK cell functional anergy and apoptosis (Das *et al.*, 2000). There is limited information about the recognition structures on the surface of NK cells and target cells.

Girardi *et al.* (2001) and Diefenbach *et al.* (2001) identified the activating receptor for NK cells, and T cells expressing the $\gamma\alpha$ or $\delta\beta$ TCR, as NKG2d on target cells. The authors characterised two NKG2d ligands as MICA and MICB in human cells. These were identified as nonclassical MHC molecules whose expression was induced by

classical stress stimuli (e.g. heat shock, mechanical stress, etc.) (Girardi *et al.*, 2001; Diefenbach *et al.*, 2001). Murine NKG2d binds to retinoic acid inducible gene family products such as Rae-1 α δ and the H60 gene product (Girardi, *et al.*, 2001, Diefenbach, *et al.*, 2001). Rae-1 and H60 also appear to be upregulated in several tumours. Cerwenka *et al.*, (2000) reported that, upon induction of these ligands on mouse tumours not normally expressing Rae-1 α or H60, NK cell susceptible tumours were rejected. Tumours that expressed these ligands but still survived in the host maintained a balance between the expression of activating versus inhibitory ligands, resulting in the dominance of immunological tolerance to tumour cells (Cerwenka *et al.*, 2000).

3.1.5.4 Role of B cells

B cells constitute 5 to 15% of blood lymphocytes and are morphologically indistinguishable from T cells. The human body has the genetic ability to recognise 10^7 - 10^9 different epitopes. Therefore, we have 10^7 - 10^9 distinct clones of B-lymphocytes, each with a unique B cell receptor. They circulate in the bloodstream in a resting state and undergo rapid proliferation after encountering antigens. Mutations at any stage of a B cell's life cycle can lead to uncontrolled proliferation giving rise to lymphomas or leukemias. B cells infiltrating cancer tissues produce IgG recognising a common tumour antigen (Imahayashi *et al.*, 2000). In addition to tumour-associated Ab responses, circulating antibodies against several other self-antigens have also been described in cancer patients (Cote *et al.*, 1983).

3.1.5.5 Role of macrophages

Macrophages mediate both specific and non-specific killing of tumour cells (Ralph *et al.*, 1991). Macrophage-mediated cytotoxicity is most efficient with cell-to-cell contact, but several soluble factors that cause cytotoxicity have also been found in macrophage culture supernatants (Currie and Basham 1978; Nathan *et al.*, 1979) (Fig. 3.2). Activated macrophage cytotoxicity against tumour cells was dependent on nitrite ion synthesis, and its inhibition led to defective cytotoxic potential (Mittra *et al.*, 2002). The effect is primarily mediated by nitric oxide and inhibits mitochondrial respiration, prevents DNA replication and inactivates various essential enzymes in target cells. Other antitumour products secreted by macrophages include TNF,

hydrogen peroxide (H₂O₂) and shed FasL (CD95L), which are equally essential for macrophage cytotoxicity (Hibbs *et al.*, 1987).

Tumour-associated macrophages (TAMs) are present in 0–80% of tumour tissues, the average being 20–30% (Tanaka *et al.*, 1995). There are several fold more macrophages in an average-sized tumour than the total number of macrophages in a normal mouse (Lee *et al.*, 1995). In addition, a tumour can cause marked qualitative changes in the subpopulations of macrophages present that have distinctive, and sometimes mutually antagonistic, functions (Eccles *et al.*, 1974). The local and systemic influences of tumour-derived factors (e.g. MHC class I expression and microenvironmental influences, such as O₂ tension) are crucial in determining the nature and number of macrophages within tumours (Bottazzi *et al.*, 1983). This could be one of the reasons why the nature of the tumour–macrophage relationship varies so considerably within tumours (Mitra *et al.*, 2003).

Macrophages are also a source of cytokines that regulate the T cell response. Due to the fact that monokines such as IL-12 synergise with costimulatory molecules B7.1 and B7.2 to enhance T cell proliferation (Murphy *et al.*, 1994), macrophages are a major factor in driving T cell expansion and differentiation. The role of host macrophages in AK-5 tumour regression has been determined by subcutaneous transplantation of AK-5 tumour cells, which led to the influx of macrophages into the peritoneal cavity and macrophage hyperactivation (Bhaumik *et al.*, 2001). Subsequent depletion of activated macrophages from the peritoneal cavity and their migration to the tumour site coincided with tumour regression (Bhaumik *et al.*, 2001). This could be due to the secretion of monocyte chemotactic substances (IL-10, TGFβ, prostaglandin E₂) by tumour cells or extracellular matrix proteins, which attract and activate macrophages directly (Alleva *et al.*, 1994).

3.1.5.6 Role of Dendritic Cells

Dendritic Cells (DCs) function as antigen presenting cells (Figure 3.2). The presence of DCs in tumour infiltrates is a good prognostic factor. The interaction between DCs and the tumour results in the release of antitumour cytokines (Becker, 1993). Patients with stage III squamous cell carcinoma exhibited a significantly better survival after

radiation therapy, when infiltrating Langerhans cells were observed in tumour tissues, due to stimulation of T cell-mediated antitumour activity (Nakano *et al.*, 1993).

Inhibition of metastasis formation by a tumour may depend on the entry of DCs into the tumour and the release of NO and other inhibitory cytokines by DCs. Conversely, a failure of DCs to enter tumours may be due to the ability of the tumour to produce cytokines that inhibit NO production (Becker, 1993). Ridge *et al.* (1998) demonstrated that antigen-pulsed DCs could directly sensitise T cells and stimulate the development of antigen-specific immune responses, including both protective and therapeutic antitumour responses. DC maturation and activation is mediated by the interaction between CD40 on DCs and CD40L on antigen stimulated T_H cells (Ridge *et al.*, 1998).

3.1.5.7 Role of Cytokines

Cytokines regulate normal cell behaviour and play an important role in the host immune response against a wide variety of infections and cancers. The activity of T_H cell populations and their cytokine products significantly influence the characteristics of the immune response to tumours. For example, T_H1 cells preferentially secrete IFN- γ and IL-2 and are responsible for cell mediated immunity, whereas T_H2 cells produce IL-4, IL-5, IL-6 and IL-10 and enhance the humoral response. A T_H3 subset, producing high levels of TGF- β with varying amounts of IL-4 and IL-10, has also been identified (Chen *et al.*, 1994). T_H3 cells have an immunosuppressive phenotype in experimental models of oral tolerance and autoimmunity (Miller *et al.*, 1992). There is an antagonistic effect between T_H1 and T_H2 cell populations as the cytokines produced by one population nullify the proliferation and function of cells of the other type. These T_H cell populations and their cytokine products thus play a decisive role in the progression or regression of a tumour.

The ability of cytokines to induce regression of a tumour is exemplified by the AK-5 tumour model, where IL-2, IL-12 (Nakano *et al.*, 1993), IFN- γ (Becker, 1993) and TNF- α are found at high levels in tumour regressing animals (Mitra *et al.*, 2003). Animals regressing the tumour show a predominant CD4/T_H1 type of cytokine response and tumour regression is primarily NK cell mediated (Ridge *et al.*, 1998).

NK cells are unable to kill AK-5 tumour cells unless they are activated with IL-2, IL-12 and IFN- γ (Khar *et al.*, 1996). Cytokine levels at the tumour site were several fold higher than in the circulation, suggesting their importance in the maintenance of tumour infiltrating lymphocytes in an activated state against the tumour (Bright *et al.*, 1995).

3.1.5.8 Role of IgG

IgG₁ is the most widely used antibody isotype for the therapy of cancer because it activates human complement, recruits NK cells for ADCC and has an extended half-life in plasma. However, Dechant and colleagues suggested that IgA also recruits cytotoxic cells in humans and may have the additional advantages of forming natural dimers with improved signalling capacity to immune cells (Dechant *et al.*, 2001).

3.1.6 Apoptosis

Apoptosis is a significant part of the cellular turnover as antibodies can kill nucleated cells by inducing apoptosis. Cells that have to be eliminated without inflammation enter apoptosis. Cross-linking of the Fas receptor (CD95) on the target cell membrane by its ligand, FasL, or with anti-Fas antibodies, induces apoptosis of the cells by the activation of caspases (Timmer *et al.*, 2002).

Fas is a cell-surface expressed member of the TNF receptor superfamily and once engaged by its ligand FasL, mediates apoptosis of the Fas-expressing cells (O'Connell, 2001; Walker, *et al.*, 1997). Some tumour cells develop 'counterattack' mechanisms of immune escape by expressing FasL (Radfar *et al.*, 2000; Khar *et al.*, 1998; O'Connell *et al.*, 1996). These tumour cells resist Fas mediated T cell cytotoxicity by expressing functional FasL and mediate the peripheral deletion of tumour-reactive T cell clones (Mitra *et al.*, 2003). Induction of Fas-mediated apoptosis by anti-Fas antibodies has been demonstrated in solid tumours implanted in mice. Unfortunately, treatment with anti-Fas antibodies or FasL has also been shown to cause severe damage to the liver (Timmer *et al.*, 2002).

3.1.7 Complement dependent cytotoxicity

The complement system consists of more than 20 plasma proteins that are proteases. These enzymes cleave each other in succession to activate and regulate the complement cascade. There are three recognised routes leading to the activation of the complement system, the classical, alternative and the lectin pathways (Eggleton *et al.*, 2000). The classical and alternative pathways are complex and illustrated in simplified form in Figure 3.4.

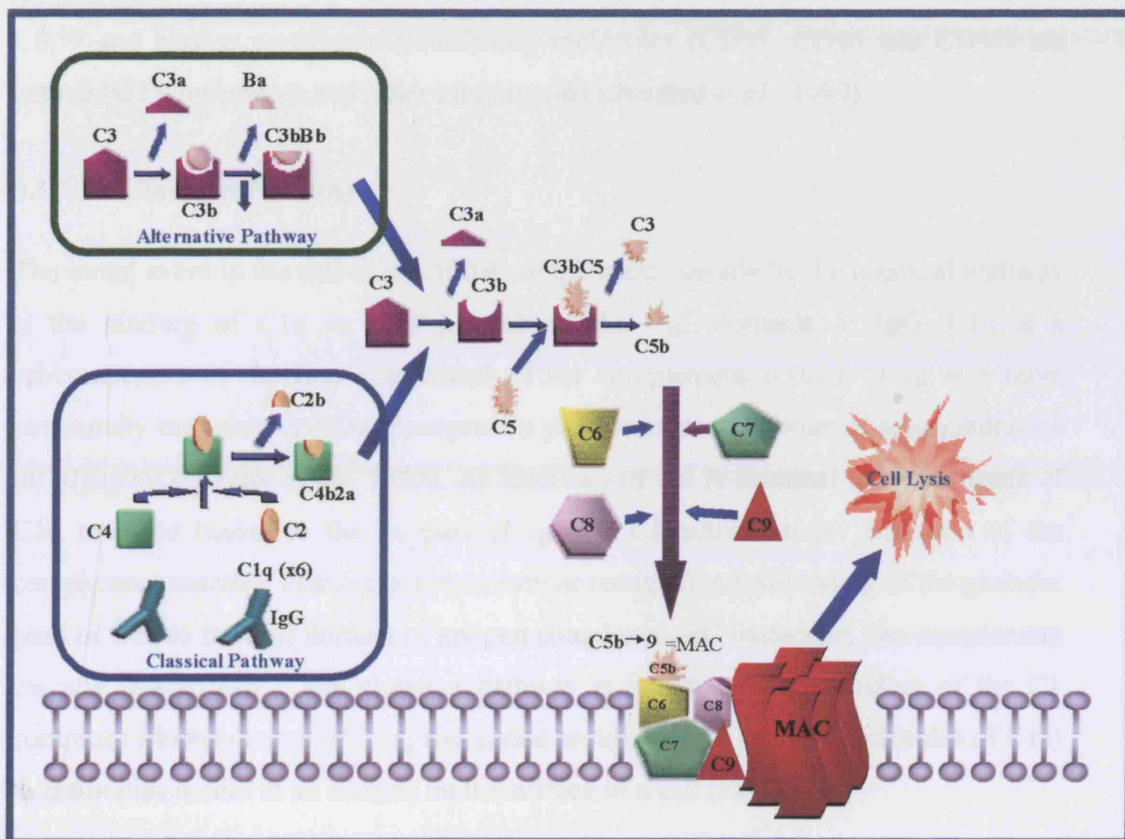


Figure 3.4: The classical and alternative pathways of the complement cascade

Complement dependent cytotoxicity (CDC) is triggered by the recognition and binding of complement fixing antibodies IgM, IgG₁, IgG₂ or IgG₃ to tumour specific surface antigens followed by complement activation. The other roles of these antibody isotypes are shown in greater detail in Table 1.9 Chapter 1.

There are three pathways of complement activation. All three are initiated by different mechanisms but converge at the point that cleaves C3 to C3a and C3b, the pivotal step in complement activation. Serum bactericidal activity is induced when the complete complement cascade (C1 to C9) is activated.

The formation of the membrane attack complex (MAC) is the final result of activation of the classical or the alternative pathway of complement. This leads to a conformational change in C9. More C9 molecules join the assembly to form a ring of C9 molecules. This is the MAC. CD59 inhibits the assembly of C9 into the MAC. CD59 and similar complement inhibitory molecules (CD35, CD46 and CD55) are expressed by melanoma and other tumours (Weichenthal *et al.*, 1999).

3.1.7.1 Classical Pathway

The initial event in the activation of the complement cascade by the classical pathway is the binding of C1q to sites present on the C_H2 domains of IgG. C1q is a subcomponent of the first component of the complement system. C1q is a large structurally complex 410kDa glycoprotein present in human serum at a concentration of 70 µg/ml (Cooper *et al.*, 1985). At least two of the N-terminal globular heads of C1q must be bound to the Fc part of Igs for C1 activation for initiation of the complement cascade. Following the molecular recognition and binding of the globular head of C1q to the C_H2 domain of antigen complexed IgG molecules, the complement cascade is activated. The classical pathway is initiated by the binding of the C1 complex (which consists of C1q, two serine proteases, C1r and two molecules of C1s) to antibodies bound to an antigen on the surface of a cell (Figure 3.5).

C1s attracts and binds C4, cleaving C4 into two active fragments, C4a and C4b. C4a fragments are lost to the serum and their ultimate fate is unknown. C4b has an active site that can bind to a cell surface receptor. C4b binding has a short half-life, and only approximately 10% of the cell bound fragments remain bound. A single C1s unit may cleave many C4 units producing a shower of C4b fragments. Bound C4b fragments attract and bind C2. Binding causes cleavage of C2 into two fragments, C2a and C2b. C2b is lost into serum and disappears. C2a remains bound to C4b forming the active complex C4b2a. The C4bC2a complex is also known as C3 convertase. It causes

cleavage of C3 into fragments C3a and C3b. C3a is released into plasma and acts as an anaphylatoxin. C3b binds to the cell surface. C4b2a can cleave many molecules of C3 enzymatically. This acts as an amplification mechanism. C3b fragments bound next to C4b2b can cause cleavage of C5. Other bound molecules of C3b are opsonic.

The C3b fragments have the greatest biological importance of the C fragments. C4b2b3b cleaves C5 into two fragments, C5a and C5b. C5a is released into serum and promotes chemotaxis and phagocytosis. C5b remains attached to the complex. C6 and C7 bind to C5b. C8 binds to the C5bC6C7 complex. C9 binds to C8 and lysis occurs. The membrane damaged by complement becomes semipermeable. Small intracellular molecules leak out. Water ions, and other molecules flow into the cell causing it to swell and burst.

Sequential activation of the components of the complement system ultimately lead to the formation of the membrane attack complex (MAC) which forms transmembrane pores that disrupt the osmotic barrier of the membrane and lead to osmotic lysis of tumour cells (Wang *et al.*, 2000a).

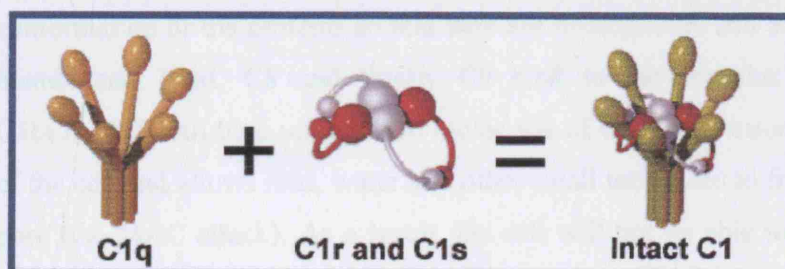


Figure 3.5: The formation of the C1 complex from the C1q, C1r and C1s components of the classical pathway. Adapted from Graille *et al.* (2000).

3.1.7.2 Alternative Pathway

Unlike the classical pathway, activation of the alternative pathway is not dependent upon antibodies, although there are indications for an enhancing activity of antibodies in the alternative pathway. The MAC (C5b-9) is inserted into the membrane of target cells and these are killed by the same lytic mechanism as in the classical pathway. Opsonisation of microorganisms by complement components (C3b, iC3b and C3dg) may also result in phagocytosis.

The alternative pathway is initiated by the covalent binding of a small amount of C3b to hydroxyl groups on cell-surface carbohydrates and proteins and is activated by low-grade cleavage of C3 in plasma. C3b binds factor B, a protein homologous to C2, to form a C3bB complex. Factor D cleaves factor B bound to C3b to form the alternative pathway C3 complex C3bBb. The binding of properdin stabilises this enzyme. The alternative C3 convertase enzymes cleave many more molecules of C3 to C3b, which bind covalently around the site of complement activation. Some of this C3b binds to the C4b and C3b in the convertase enzymes of the classical and alternative pathways, respectively, forming C5 convertase enzymes. C5 convertase is C4b2a3b for the classical and 3bBb3b for the alternative pathway. C3b acts as an acceptor site for C5, which is cleaved to form the anaphylatoxin C5a and C5b, which initiates the formation of the membrane-attack complex.

C5 is broken down into C5a and C5b after binding to C5 convertase. C5a diffuses away and has anaphylatoxin activity including vasodilation and chemotaxis. The C5b fragment binds directly to the target organism (or cell) and becomes the binding site for other complement components. C5b will quickly degrade unless C6 binds to it creating C5bC6, to which C7 will then bind. The addition of C7 changes the conformation of the proteins so that they are hydrophobic and able to insert into lipid membranes. Next, C8 and finally C9 bind to the complex creating the MAC. C5bC6C7C8 will bind up to 10-16 molecules of C9. MAC inserts into the membrane of the cell and allows ions, water and other small molecules to freely pass through the pore (the MAC attack). As a result, the cell will not be able to maintain osmolarity and therefore will quickly die.

3.1.7.3 Lectin Pathway

The lectin pathway is illustrated in Figure 3.6. Activation of the lectin pathway is mediated by mannose-binding lectin (MBL, a C1q homologue), a pattern-recognition receptor specific for microbial carbohydrates. MBL is associated with the MASP1 and MASP2 proteins (MBL associated serine proteases 1 and 2). Binding of the MBL to its microbial ligand activates the proteases, which leads to the cleavage of the complement components C2 and C4. The cleavage products C2a and C4b then form a C3 convertase, which initiates the complement cascade by cleaving the C3 protein.

The complex of MBL and its proteases function similarly to the C1 complex of the classic complement cascade. It is important to note, however, that the C1r and C1s serine proteases are activated by the binding of C1q to the antibody–antigen complex and are thus dependent on antibody responses, whereas activation of the lectin complement pathway is triggered directly by microbial recognition and is therefore independent of the adaptive immune response (Medzhitov *et al.*, 2000).

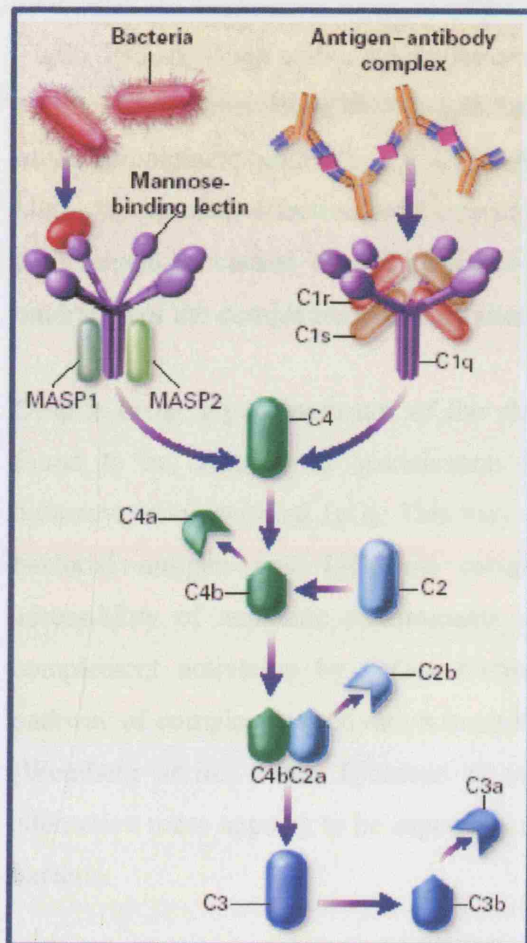


Figure 3.6: The Lectin and classical pathways of the complement cascade. Both pathways lead to activation of the C4 component and the subsequent formation of the membrane attack complex and hence lysis (Medzhitov *et al.*, 2000).

The role of the lectin pathway is to trigger inflammation ($C5a > C3a > C4a$), chemotactically attract phagocytes to the infection site ($C5a$) and to promote the attachment of antigens to phagocytes via enhanced attachment or opsonisation ($C3b > C4b$). Furthermore, it serves as a second signal for the activation of naive B-lymphocytes ($C3d$), causes lysis of gram-negative bacteria and human cells displaying

foreign epitopes (MAC) and removes harmful immune complexes from the body (C3b>C4b).

3.1.8 Role of IgG in Complement Activation

The structural diversity in the hinge region of IgG subclasses gives rise to differences in their ability to activate complement. The capacity of the four human IgG subclasses (in monomeric form) to bind C1q decreases in the order: IgG₃ > IgG₁ > IgG₂ > IgG₄. IgG₄ does not activate complement (Flanagan *et al.*, 1982; Van Loghem *et al.*, 1986). Hinge-dependent Fab-Fab and Fab-Fc flexibility determines the accessibility of the complement binding site to C1q. IgG₃, the human IgG subclass with the longest hinge, is the most effective complement activator. The inability of IgG₄ to activate complement is caused by the structure of the Fc fragment as well as the steric hindrance of the complement binding sites by the Fab arms.

Despite being a poor activator of the classical complement cascade IgG₂ has been found to be effective in opsonisation and killing of certain bacterial strains in bioassays using purified IgG₂. This may be explained by a combined effect of both bacterial antigen and IgG₂ on complement activation. Epitope density and accessibility of antigenic determinants on the bacteria may determine the actual complement activation by IgG₂. Alternatively, IgG₂ may utilise the alternative pathway of complement activation in an Fc-independent way, bypassing C1q binding (Weinberg *et al.*, 1986; Bjornson *et al.*, 1987). Complement activation via the alternative route appears to be especially important in the opsonisation and killing of bacteria.

3.1.9 Opsonisation and Induction of Phagocytosis

Most microorganisms are incapable of activating complement or of binding phagocytes directly. Antibodies act as flexible adapters mediating the adherence of infectious agents to phagocytes. The antibody forms a bridge that attaches the microbe to the Fc receptor on the phagocyte cell surface.

3.1.10 Decay Accelerating Factor (CD55)

Complement Decay Accelerating Factor (DAF) or CD55 is a member of a family of proteins that inactivate complement. CD55 inhibits the complement cascade by inactivating C3 convertases and preventing C3b deposition on cell membranes. C3b is a powerful stimulus of phagocytosis by antigen-presenting cells and also initiates the formation of the membrane attack complex (Muller-Eberhard *et al.*, 1988).

CD55 can also transduce signals in lymphocytes and is a ligand for CD97 that is expressed by activated T cells (Hamann *et al.*, 1996). Spendlove *et al.* (1999) published a study suggesting that CD55 plays a role in signalling between the innate and adaptive immune responses. This makes it a target for therapy as absence of the molecule makes the tumour cells susceptible to lysis by complement (Spendlove *et al.*, 1999).

Cross-linking with anti-CD55 monoclonal antibodies can induce T-cell proliferation and signal transduction via p56^{lck} and p59^{fyn} (Shenoy-Scaria *et al.*, 1992; Davis *et al.*, 1988). Furthermore, cross-linking of the complement regulatory protein CD46 results in down-regulation of interleukin-12 production by antigen-presenting cells. Over-expression of CD55 by tumour cells can make the tumour more resistant to NK lysis as shown for over-expression of CD55 on K562 tumour cells (Finberg *et al.*, 1992).

3.1.11 Complement Regulatory Proteins

CD46 and CD59 are two membrane-associated complement regulators that also inhibit C3b deposition and assembly of the membrane attack complex (Liszewski *et al.*, 1996). These proteins are overexpressed by a range of solid tumours and have been implicated in the limited success of antibody-mediated lysis of tumour cells (Juhl *et al.*, 1997). However, their role in inhibition of antigen processing by tumours is unknown. The three complement regulatory proteins share a similar structure comprising four SCR (short chromosome repeat) domains. CD55 and CD59 are GPI anchored, whereas CD46 has a classical transmembrane domain (Lublin *et al.*, 1989). They have all been implicated in signal transduction in T cells (Spendlove *et al.*, 1999).

3.1.12 Complement Resistance

The mechanisms underlying complement resistance in malignant cells have only recently been understood. Information on resistance mechanisms is important for development of immunotherapies for solid tumours with mAbs. IgG subclasses differ in their ability to activate the complement system and to mediate Ab-dependent cellular cytotoxicity (ADCC) (Asghar *et al.*, 1995). In addition, complement fixation can induce a strong inflammatory response, which can initiate other anti-tumour immune mechanisms.

Tumour cells often escape lysis since the complement system cannot discriminate efficiently between foreign and self. Cells express one or more of a group of molecules known as membrane bound complement-regulatory proteins (mCRPs) that regulate complement activation on the cell surface (e.g. CD35, CD46, CD55 and CD59) (Morgan *et al.*, 1998). With the exception of CD35, these regulators are also expressed on most solid tumours. Soluble complement inhibitors can also inhibit the complement attack against tumours.

The complement system undergoes continuous low-level activation within the circulation, thereby patrolling for potential invaders (Liszewski *et al.*, 1996). Whether this surveillance function also extends to tumour cells escaping into the blood from their original site has not been examined thoroughly, although, in principle, the complement system has the ability to destroy such nucleated cells (Mitra *et al.*, 2003).

3.1.13 Complement Deficiencies

The infections to which patients with inherited deficiencies of complement are susceptible are determined by the role that the specific deficient component has in the normal host defence. For example, patients with a deficiency of C3, or one of the components of the classical or alternative pathway that are responsible for activating C3, have an increased susceptibility to organisms for which C3b-dependent opsonisation plays an especially important role. These include organisms such as the *pneumococci*, *streptococci*, and *H. influenzae* type b. Conversely, patients with deficiencies of C5, C6, C7, C8 or C9 are able to generate opsonically active C3b and thus have no increased susceptibility to infection by these organisms (Loos *et al.*,

1986; Johnston *et al.*, 1969). However, because they lack the ability to generate C5 through C9-dependent bactericidal activity, they are especially susceptible to *Neisseria* species, for which bactericidal activity is a critical host defence mechanism (Nicholson *et al.*, 1979).

Factor I is an inhibitor of the alternative pathway C3-cleaving enzyme C3bBb. As a result, patients with factor I deficiency have uncontrolled continuous activation of C3, with a resulting secondary serum C3 deficiency (Abramson *et al.*, 1971; Thompson *et al.*, 1977). Factor I-deficient patients have increased susceptibility to infection with encapsulated bacteria to which C3-deficient patients are susceptible.

A variety of defects in host defence have been identified in neonates and each one could be a contributor to an increased susceptibility to infection with a wide range of bacteria, viruses and fungi. Among the defects are those in the complement system (Johnston *et al.*, 1979). The serum levels of most of the individual components in healthy infants are reduced to between 50% and 80% of the levels found in adults. Premature infants have even lower complement levels. In addition, their ability to activate C3 and C5 is reduced resulting in a decrease in serum opsonising and chemotactic activities (Winkelstein *et al.*, 1979).

Patients with sickle cell disease are more susceptible to infection, most notably bloodborne infections, such as septicaemia and meningitis caused by *S. pneumoniae* (Robinson *et al.*, 1966). There are two defects in the host defence that contribute to the increased susceptibility to infection in these patients. One of these is functional, or anatomic, asplenia (Pearson *et al.*, 1969) and the other a decrease in serum opsonising activity (Winkelstein *et al.*, 1968). A number of studies have shown that the defective serum opsonising activity is secondary to defective activation of the alternative pathway (Johnston *et al.*, 1973) although the exact basis for the defect in alternative pathway activation is unknown. Both the asplenia and the serum opsonising defect contribute to their increased susceptibility to infection because patients who have had a splenectomy alone, such as traumatic splenectomy, are not nearly as susceptible to systemic pneumococcal infections as children with sickle cell disease (Wilson *et al.*, 1979).

Children with the idiopathic nephrotic syndrome have an increased susceptibility to pneumococcal peritonitis and sepsis (Churg *et al.*, 1970). They are at greatest risk when they are in relapse and large amounts of protein, such as factor B, are found in their urine. This results in reduced serum opsonising activity, but when factor B is added back to their serum, the defect is corrected (McLean *et al.*, 1977). This suggests that loss of factor B in the urine is responsible for their deficient opsonising activity and, thus, may contribute to their increased susceptibility to infection.

3.1.14 Role of Complement in Cancer

Although natural antibody responses against cell surface antigens have been detected in cancer patients only few studies have indicated that complement is inherently activated in tumours *in vivo* (Irie *et al.*, 1974). Some strategies have been designed to target and activate complement against tumour cells. Reiter *et al.* (1989) published one such study using heteroconjugates of monoclonal antibodies and C3b or cobra venom factor (CVF) to activate the alternative pathway by replacing C3b in the C3 convertase. The CVFBb complex was found to be insensitive to complement inhibitors and amplified the *in vitro* kill of tumour cells by complement from 10-15% to 70-100% (Reiter *et al.*, 1989). Yefenof and colleagues investigated interferon in an effort to amplify the ability of tumour cells to activate complement via the alternative pathway. The results showed that interferon-treated tumour cells fixed C3b to their surfaces through the alternative pathway (Yefenof, *et al.*, 1985).

CDC is believed to be an important action mechanism for the anti-CD20 monoclonal antibody used therapeutically in the treatment of lymphoma patients. B cell lymphoma patients express CD46, CD55 and CD59 molecules at various levels on their malignant cells. This results in differences in sensitivity of cells to complement-mediated lysis. Blocking CD55 and CD59 activity in patients responding poorly to mAb therapy resulted in 5 to 6 fold increased lysis of tumour cells (Golay *et al.*, 2001).

Wang *et al.* (1999) tested two anti-melanoma immunoconjugates consisting of human scFvs fused to the Fc part of human IgG₁. The authors showed that the

immunoconjugates can specifically target human melanoma cells for lysis by NK cells and complement (Wang *et al.*, 1999).

3.1.15 Protein A

This chapter examines the potential of scFv fusion proteins to *Staphylococcus aureus* protein A (SpA) for tumour therapy *in vitro*. SpA is a component of the cell wall structure of *Staphylococcus aureus* and has been used for the isolation, purification and detection of antibodies (Sinha *et al.*, 1999).

Protein A is a type I membrane protein bound to the cell wall via its C-terminal cell-wall binding region. It is a 42 kDa polypeptide that binds to the Fc fragment of IgG from various species (Forsgren *et al.*, 1966). It is a monomeric protein lacking cysteine residues and is stable in the range of pH 1.0 to 12.0. The isoelectric point is 4.85–5.10. It binds residues on the surface of C_{H2} and C_{H3} domains of immunoglobulin heavy chains (Langone, 1982).

SpA consists of five highly homologous extracellular Ig-binding domains in tandem (E, D, A, B and C). Protein A exists in both secreted and membrane-associated forms. It has two distinct Ig-binding activities. Each domain can bind Fc γ (the constant region of IgG that exerts effector functions) and Fab (the Ig fragment mediating antigen recognition) (Nilsson *et al.*, 1987). The Fc γ binding site has been localised to the elbow region at the C_{H2} and C_{H3} interface of most IgG subclasses. This binding property has been extensively utilised for labelling and purification of antibodies (Deisenhofer *et al.*, 1981; Tashiro *et al.*, 1995).

The Fab specificity is less well characterised but has been shown to involve a site on the variable region of the Ig heavy chain (Vidal *et al.*, 1985). The Fab binding specificity is restricted to antibodies with a human variable region Kabat subgroup 3 (Sasso *et al.*, 1989; 1991; Sasano *et al.*, 1993) and their homologues of other mammalian species (Seppala *et al.*, 1990; Cary *et al.*, 1999). *In vitro* stimulation with SpA can select B cells that secrete these antibodies and promote production of antibodies that may include rheumatoid factor autoantibodies (Kristiansen *et al.*, 1994; Kozlowski *et al.*, 1995). *In vivo* exposure to recombinant SpA can result in

supraclonal suppression and deletion of B lymphocytes that are susceptible depending on their V_H usage (Silverman *et al.*, 1998; Cary *et al.*, 2000).

Although the mechanism(s) are not defined, experimental models indicate that SpA enhances staphylococcal virulence (Foster *et al.*, 1988; Patel *et al.*, 1987). Interactions of SpA with host B lymphocytes are similar to superantigens and T lymphocytes causing a variety of inflammatory diseases, including toxic shock syndrome and food poisoning (Kotzin *et al.*, 1993; Bohach *et al.*, 1990; Papageorgiou *et al.*, 1998). T cell superantigens have been postulated to contribute to the pathogenesis of autoimmune diseases (Kotzin *et al.*, 1993; Li *et al.*, 1999). These superantigens target T-cell receptors (TcRs) from particular variable β chain (V _{β}) families and can induce global changes in T lymphocyte repertoires (Kotzin *et al.*, 1993).

The interaction between SpA and IgG from a number of mammalian species is well documented (Kronvall *et al.*, 1971). Table 3.1 summarises the interaction of Protein A with Ig from various species. SpA binds to the C_{H2} and C_{H3} domains of the Fc region of antibodies and with lower affinity to some V_H domains of the human V_{H3} family and its mouse homologues (Forsgren *et al.*, 1966). Protein A binding has been studied by NMR and was shown to bind the V_{H3} surface between the antigen binding site of the antibody and bottom of the V_H domain (Jansson *et al.*, 1998) as well as the interphase of C_{H2} and C_{H3}. High and low affinity FcR binds the antibody hinge and the upper part of C_{H2} (Kato *et al.*, 1995). However, it should also be noted that the 'Z' domain has a much lower affinity for the V_{H3} than the parental B domain.

C1q binding has been studied by mutagenesis and was shown to bind to the C_{H2} domain between the protein A and FcR binding sites. Simultaneous binding of an antibody to protein A and C1q or FcR should be possible (Wines *et al.*, 2000) although some steric hindrance could occur. The FcR binding site on antibodies is well apart from the protein A binding site and therefore there should be no steric hindrance with IgG binding to protein A and FcR.

It is well established that SpA is a potent biological response modifier, showing immunomodulatory properties, including antitumour, antitoxic, anticarcinogenic, antifungal and antiparasitic activities (Sinha *et al.*, 1999). Protein A can act as B and

T cell mitogen (Scouros *et al.*, 1983; Ford *et al.*, 1985). Protein A significantly elevated T_H1 cytokines, e.g., IFN- γ , TNF- α but also IL-1 α , IFN- γ , IL2, IL4, IL6, IL10 (Sinha *et al.* 1999b) regulating cellular growth and differentiation (Taniguchi *et al.* 1995) on one hand and apoptosis (Geng *et al.* 1996) on the other. Protein A stimulates generation of nitric oxide (NO) from murine peritoneal macrophages which in turn induces cytotoxic damage to the tumour cells by apoptosis (Chattopadhyay *et al.*, 2002).

Ghosh *et al.* (1999) reported that protein A stimulates specific lymphocytes to act as potential 'immunorestorers'. The authors showed that the percentage of various cells bearing different clusters of differentiation markers, e.g., CD4⁺, CD8⁺, CD19⁺, increases considerably after inoculation of mice with protein A. In addition, CD34⁺ progenitor cells in the bone marrow increased significantly upon protein A treatment. This suggests the use of protein A therapy in diseases with immunosuppression or as an adjuvant therapy as an immunorestorer together with toxic drugs causing immunosuppression. Stimulation of macrophage number and macrophage function by protein A may be related to its antitumour activities (Prasad *et al.*, 1987).

It is possible that in order to exert biological function large molecular structures may not be required. The biological function of protein A may be mediated through a portion of the whole molecule such as the active site or by a proteolytically generated small peptide derivative (Sinha *et al.*, 1999). This has also been demonstrated to be the case for the different domains of protein A (Sengupta *et al.*, 1999). Initial binding studies on the formation of protein A-IgG soluble complexes appeared to show that the five domain SpA molecule has only four functional IgG binding domains (B, D, A and C) (Hanson *et al.*, 1984). This suggested that the E domain is non-functional. However, subsequent studies showed that all 5 protein A domains are functional (Moks *et al.*, 1986; Jansson *et al.*, 1998).

Species	Immunoglobulin	Binding- Protein A
Human	IgG (normal)	++++
	IgG ₁	++++
	IgG ₂	++++
	IgG ₃	-
	IgG ₄	++++
	IgM	-
	IgA	-
	IgE	-
	IgD	-
	Fab	++
	K light chains	-
	L light chains	-
	ScFv	++
Mouse	IgG ₁	+
	IgG _{2a}	++++
	IgG _{2b}	+++
	IgG ₃	++
Rat	IgG ₁	-
	IgG _{2a}	-
	IgG _{2b}	-
	IgG ₃	+
Bovine	IgG	++
Cat	IgG	++++
Chicken	IgG	-
Dog	IgG	++++
Goat	IgG	+/-
Guinea pig	IgG	++++
Hamster	IgG	+
Horse	IgG	++
Pig	IgG	+++
Rabbit	IgG	++++
Sheep	IgG	+/-
Cow	Ig	++
Koala	Ig	-
Llama	Ig	-

Table 3.1: Protein A interaction with various species and IgG regions. ++++ denotes the highest affinity for binding whereas +/- shows very weak or no binding (adapted from SIGMA and Amersham Biosciences catalogues).

A variant of the B domain of SpA is the artificially created 'Z' domain (Nilsson *et al.*, 1987). The Z domain of protein A is an anti parallel three-helix bundle of 72 residues, which binds to the Fc portion of human immunoglobulin IgG₁ but not to V_H3 domains (Braisted *et al.*, 1996). Figure 3.7 illustrates the structure of the Z domain with four

types of side chains. Helix I binds the Fc and helices II and III bind V_H3 (Graille *et al.*, 2000) in protein A, but not in 'ZZ'.

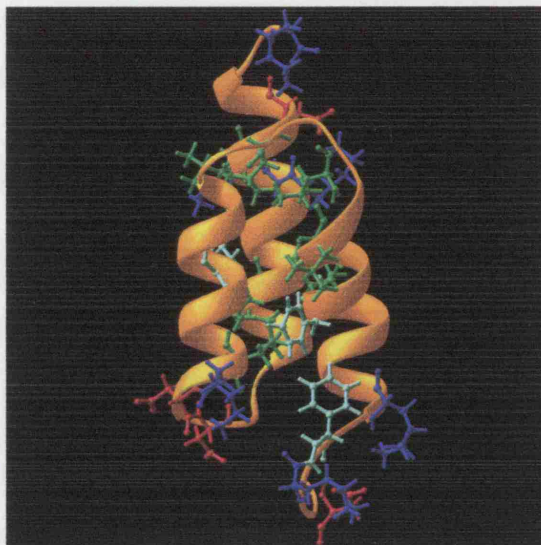


Figure 3.7. The artificial Z domain with four types of side chains. Lysine (K) is blue, phenylalanine (F) is aqua, leucine (L) is green and aspartic acid (D) is red. This domain of the protein contains no disulphide bonds and is 72 amino acids long (<http://www-nmr.cabm.rutgers.edu/>).

3.1.15.1 Use of Protein A For Cancer Therapy

Protein A of *Staphylococcus aureus* has been used in cancer treatment because of its potential for removing serum-blocking factors in patients with malignancies (Solal-Celigny *et al.*, 1985). Several reports have indicated antitumour effects from the immunoadsorption of plasma IgG from tumour bearing animals and humans with protein A (Bensinger *et al.*, 1984). This was reported by Bensinger and colleagues as the results of a phase I trial at the University of Washington. The authors used protein A chemically linked to crystalline silica for continuous immunoadsorption of plasma in patients with advanced malignancies.

The side effects observed were manageable and included chills, fever, nausea, tumour pain, hypotension and respiratory symptoms. However, the observed antitumour responses were modest. Three of seven patients with melanoma had responses less than 50%. One of three patients with breast cancer had a response less than 50%. This

study suggested the possibility of using staphylococcal protein A immuno-adsorption in patients with a more favourable disease stage.

Protein A functions *in vivo* by the formation of $[(\text{IgG})_2\text{SpA}]_2$ complexes (Das *et al.*, 1987). The use of protein A as an *ex-vivo* immunoadsorbent for immunotherapy resulted in antitumour and antiviral responses in animals (Messerschmidt *et al.*, 1988). Five of seven patients had regression of metastatic breast adenocarcinoma when treated using a column containing 50 or 200 mg protein A (Ainsworth *et al.*, 1988).

An immunoadsorption column containing highly purified protein A covalently attached to a silica matrix was used to treat patients with refractory malignancies. Only 22 of 104 patients showed an objectively measurable anti-tumour response causing a reduction in tumour burden (Snyder *et al.*, 1991).

The effects of purified protein A on induction of IL-2 activated killer (LAK) activity has been studied in melanoma patients. Singh *et al.* (1992) observed initiation of DNA synthesis in PBMCs from melanoma patients treated with SpA and IL-2. The authors concluded that the activation of LAK cells with cytotoxic potential by relatively low doses of IL-2 and protein A regime could be useful tools in the development of LAK immunotherapy for cancer patients (Singh *et al.*, 1992).

A Protein A-lymphotoxin chimera (ALT) and an amino-terminal 19 amino acid-deleted lymphotoxin (dLT) have been shown to cause tumour regression, hemorrhagic necrosis and complete regression in a nude mouse tumour model. Biodistribution studies with ^{125}I -labeled ATL and dLT showed that ALT was retained longer in the administered tumour site and cleared more slowly than dLT (Miki *et al.*, 1991). These findings suggest that a protein A fusion can exhibit improved biodistribution whilst maintaining the anti-tumour activities of the fusion molecule, in this case lymphotoxin.

Shukla *et al.* (1996) investigated the antitumour activities of protein A in Swiss albino mice in a skin carcinogenesis model. The authors showed that protein A can effectively inhibit dimethylbenz[a]anthracene initiated and TPA promoted skin carcinogenesis.

A mechanism proposed for the anti-carcinogenic activity of protein A is the induction of apoptosis in tumour cells. Protein A mediated activation of the immune system of the host triggers the release of apoptogenic factors such as tumour necrosis factor- α (TNF- α) and nitric oxide (NO). This induces apoptosis by soluble immune mediators through the up-regulation of pro-apoptotic factors (p53 and Bax) and down-regulation of anti-apoptotic factors e.g. Bcl-2 resulting in the activation of caspase-3 (Das *et al.*, 2002)

Protein A complexes with IgG have been shown to inhibit the growth of Meth A fibrosarcomas in BALB/c mice. It has been shown that potential mitogenic enterotoxin contaminants are not responsible for these effects (Das *et al.*, 1989).

A gene fusion system has been constructed for fusions to the 3' end of gene ZZ, encoding a two-domain analogue of SpA and designated ZZ (Rondahl *et al.*, 1992). This is commercially available as vector pEZZ18. Figure 3.8 shows a comparison of the five protein A domains D, E, A, C and B with the artificial Z domain.

It has been claimed that in serum, staphylococcal bacteria may bind IgG molecules in the wrong orientation on their surface and that this disrupts opsonisation and phagocytosis. It has been speculated that this is due to the bottom of the 5 protein A domain repeats pointing away from the *S. aureus* surface. This does not allow complement bound to the protein A complement cascade to reach the bacterial surface, resulting in no opsonisation and hence no phagocytosis. The mutual inhibition of the binding of C1q and protein A domains indicates that the binding sites for C1q and protein A are closely located in the C_H2 domain (Laky *et al.*, 1985). However, the potential of whole protein A to activate immune responses such as the complement system, cell mediated cytotoxicity, interferon induction, activation of polyclonal antibody synthesis and mitogenic stimulation of lymphocytes remains (Catalona *et al.*, 1981).

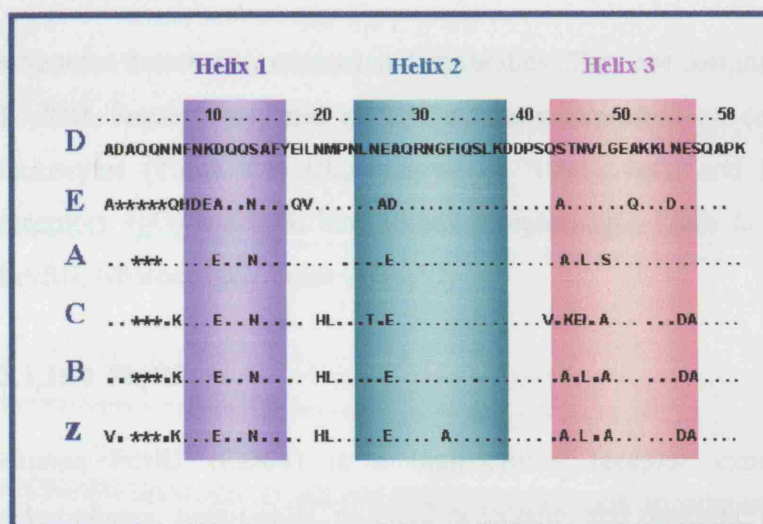


Figure 3.8: Sequence alignment of the five protein A domains and the artificially constructed Z domain. * indicates the position of deleted amino acids at the N-terminus of domains E, A, C, B and Z.

3.1.16 Interaction with Fc receptors

Phagocytosis is initiated by an interaction between the Fc fragment of immunoglobulin and its receptors. Fc receptors for IgG (FcγR) are expressed primarily on effector cells of the immune system, in particular macrophages, monocytes, myeloid cells and dendritic cells.

The four human IgG subclasses, IgA and IgE show differences in their interaction with FcRs. Binding of the Fc part of IgG to a FcγR is instrumental in the induction of the cell's effector function (Van de Winkel *et al.*, 1993). In this way, FcγRs play a key role in bridging antibody activity and cellular effector mechanisms. The latter comprise e.g. phagocytosis, endocytosis, antibody-mediated cellular cytotoxicity, release of a range of inflammatory mediators, antigen presentation and clearance of immune complexes. Moreover, since several FcγR-bearing cell types (e.g. macrophages, monocytes and dendritic cells), have the capacity to present antigens to T lymphocytes, FcγR-induced phagocytosis also plays a role in antigen presentation and amplification of the immune response. As a result, interaction between FcγR and IgG antibodies is pivotal in the immune response against infectious agents (Bredius *et al.*, 1994b).

There are three distinct types of Fcγ receptors on human leukocytes that are distinguishable by structural and functional properties, as well as by antigenic

structures detected by monoclonal antibodies. They are designated FcγRI, FcγRII, and FcγRIII, respectively, and are differentially expressed on (overlapping) subsets of leukocytes (Table 3.1) (Ravetch, *et al.*, 1991). IgG₁ and IgG₃ bind to all three receptors. IgG₂ and IgG₄ bind to one receptor. IgG₂ binds to one variant of receptor FcγRII, whereas IgG₄ binds to FcγRI.

3.1.16.1 FcγRI

Human FcγRI (CD64) is a high-affinity receptor expressed on monocytes, macrophages, neutrophils, myeloid precursors and dendritic cells. FcγRI has a high affinity for monomeric human IgG₁ and IgG₃. Its affinity for IgG₄ is almost 10 times lower and it does not bind IgG₂ or show genetic polymorphism (Ravetch *et al.*, 1991).

3.1.16.2 FcγRII

Human FcγRII (CD32) is expressed as isoforms IIa, IIb1, IIb2, IIb3 and IIc. It is the most widely distributed human FcγR type and is expressed on most types of blood leukocytes, as well as on Langerhans cells, dendritic cells and platelets. FcγRII is a low-affinity receptor that binds aggregated IgG and is the only FcγR class able to bind IgG₂. FcγRIIa shows genetics polymorphism, resulting in two distinct allotypes, FcγRIIa-H131 and FcγRIIa-R131, respectively (Ernst *et al.*, 1991; Bredius *et al.*, 1995). This functional polymorphism is attributable to a single amino acid difference: a histidine (H) or an arginine (R) residue at position 131, which is critical for IgG binding. FcγRIIa readily binds human IgG, and IgG₃ but not IgG₄. The FcγRIIa-H131 has a much higher affinity for complexed IgG₂ than the FcγRIIa-R131 allotype (Bredius *et al.*, 1995).

3.1.16.3 FcγRIII

FcγRIII (CD16) has two isoforms, both of which are able to bind IgG₁ and IgG₃. The FcγRIIIa, with an intermediate affinity for IgG, is expressed on macrophages, monocytes, natural killer (NK) cells and subsets of T cells. FcγRIIIb is a low-affinity receptor for IgG, selectively expressed on neutrophils. Studies with myeloma IgG dimers have shown that IgG₁ and IgG₃ bind to FcγRIIIb with low affinity, while no

binding of IgG₂ and IgG₄ has been found (Parren *et al.*, 1992; Huizinga *et al.*, 1989). FcγRIIb expresses a co-dominant, bi-allelic polymorphism, the allotypes being designated NA1 (Neutrophil Antigen) and NA2 (Bredius *et al.*, 1994).

3.1.16.4 Binding Sites for IgG on FcγRs

The binding sites for IgG on FcγR I, II and III all overlap. These differ in their ability to bind IgG of different isotypes and show different expression patterns on monocytes, macrophages, neutrophils, NK cells, B cells, T cells, mast cells and basophils. Serum IgG redirection by RAFT3ZZ will activate a variety of immune cells against melanoma in an effectively isotype independent manner. The affinity and specificity of FcγRs for binding human and mouse IgG and the expression of Fc receptors in hematopoietic cells is summarised in Table 3.2.

	CD	IgG Affinity (K _a)	Specificity		FcR Family							
			hIgG	MigG	MØ	Mono	Neut	NK	B	T	Mast	Baso
HuFcγRI	CD64	10 ⁸ -10 ⁹	1=3>4>>2	2A=3>>1,2b	+	+	+/i	-	-	-	-	-
MoFcγRI	CD64	10 ⁷ -10 ⁸	3>1>4>>2	2a,2b>>1,3	+	?	?	-	-	-	-	-
HuFcγRIIA	CD32	<10 ⁷	1=3>>2,4	1,2b>>2a,3	+	+	+	-	-	-	?	?
HuFcγRIIB	CD32	<10 ⁷	1=3>>2,4	1,2b>>2a,3	+	+	-	-	+	-	?	?
HuFcγRIIC	CD32	<10 ⁷	1=3>>2,4	1,2b>>2a,3	+	+	+	-	-	-/?	?	?
MoFcγRII	Ly17	9x10 ⁵	3>1>4>>2	2b>2a>1>>3	+	+	+	-	+	+	+	?
HuFcγRIIIA	CD16	<10 ⁷	1,3>>2,4	3>2a>2b>>1	+	+/i	-	+	-	+	?	?
HuFcγRIIIB	CD16	<10 ⁷	1,3>>2,4	3>2a>2b>>2	-	-	+	-	-	-	?	?
MoFcγRIIIα	CD16	<10 ⁶	3>1>4>>2	2b>2a>1>>3	+/i	?	+	+	-	-	+	?

Table 3.2: Summary of the FcR CD nomenclature, binding characteristics, ligand affinity, specificity for human and mouse IgG subclasses and the expression of the FcR in hematopoietic cells. Hu: human, mu: murine, MØ: macrophages, Mono: monocytes, Neut: neutrophils; NK: natural killer cells; B: B cells; T: T cells; Mast: Mast cells; Baso: basophils; I: inducible (adapted from Ravetch, *et al.*, 1991).

3.1.17 Hypothesis

Protein A binds most Ig isotypes and is able to mediate ADCC and CDC. Effector cells, particularly macrophages, monocytes, myeloid cells and dendritic cells have Fc receptors that interact with Ig to trigger immune responses. ScFvs distinctively bind the epitope on melanoma cells inducing a melanoma specific response.

The scFv ZZ fusion protein should recruit IgG for melanoma specific kill by ADCC and CDC (Figure 3.9). The potential advantages of scFv ZZ compared with mAbs include: their smaller size that could increase tumour penetration therefore allowing more effective tumour destruction. They can also be easier to produce (Schultz *et al.*, 2000). In addition, the protein may trigger an anti-idiotypic response.

Other advantages of using ZZ include its interaction with mouse and human IgG (Table 3.2), and its ability to bind to 2 IgG molecules effectively creating dimers with IgG complexes (Das *et al.*, 1987). It binds different isotypes for optimal ADCC and CDC. The proposed mechanisms for scFv ZZ fusion-mediated ADCC and CDC are illustrated in Figure 3.9.

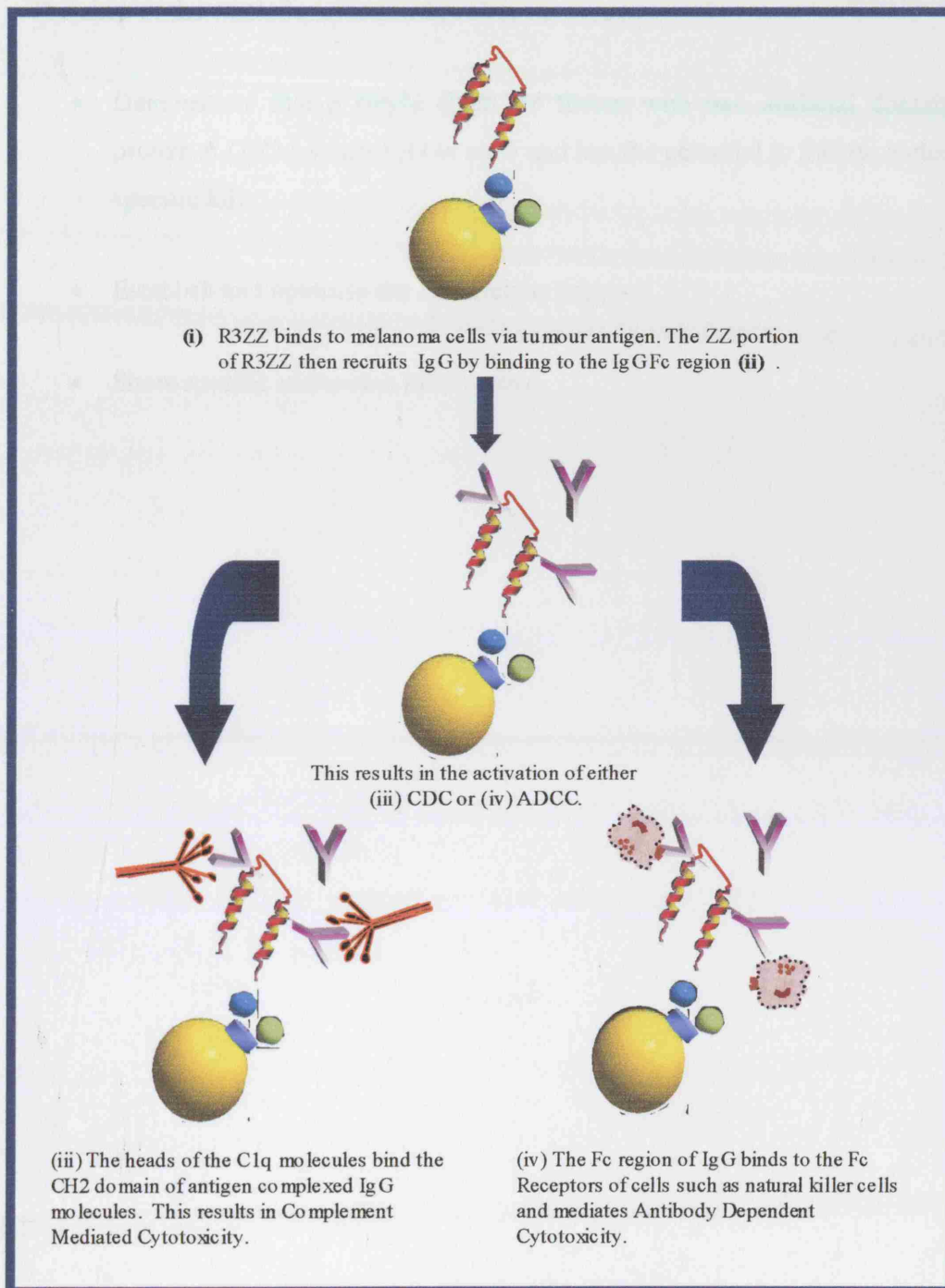


Figure 3.9: Strategy for the melanoma specific redirection of IgG by scFv ZZ fusion protein.

3.1.18 Aims

The aims of the work described in this chapter were to:

- Demonstrate that a single chain Fv fusion with two artificial domains of protein A (ZZ) recruits IgG *in vitro* and has the potential to initiate melanoma specific kill.
- Establish and optimise the cytotoxicity assays.
- Show specific melanoma kill *in vitro*.

3.2 MATERIALS AND METHODS

3.2.1 Design of RAFT3ZZ

The single-chain Fv (RAFT3) developed for *in vivo* targeting of melanoma was the most stable scFv binding the High Molecular Weight-Melanoma Associated Antigen (HMW-MAA) (Kang *et al.*, 2000) available at the time the construct was made. Therefore, RAFT3 scFv was selected for modification as a therapeutic molecule. The vector pEZZ18 commercially available from Amersham Biotech was used to clone the single-chain Fv with an N-terminal fusion to 2 ZZ domains (Nilsson *et al.*, 1987). The construct has two promoters, the IPTG inducible *lac* promoter from vector pUC18 and the constitutive protein A promoter. Therefore, expression is constitutive. In addition it has the protein A secretion leader. The design of the fusion molecule is illustrated in Figures 3.10 and 3.11.

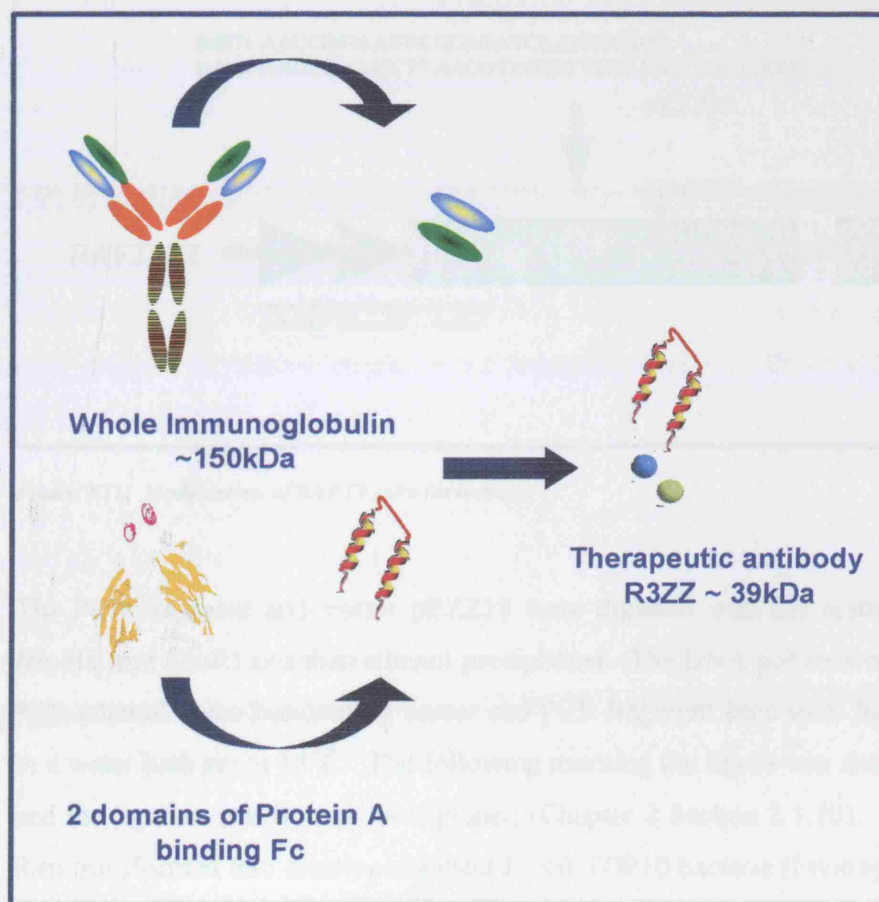


Figure 3.10: Modification of RAFT3 scFv to RAFT3ZZ for therapeutic use.

The purification and detection methods for RAFT3ZZ differ from the parent RAFT3 scFv as the former lacks the *c-myc* and hexa-histidine tail. Therefore, the IgG binding properties of protein A were utilised for both purification and detection of RAFT3ZZ. The binding properties of protein A are shown in Table 3.2.

3.2.2 Cloning of RAFT3ZZ

The RAFT3 fragment was amplified using RAFT3 DNA template and PCR primers R417 & R418 binding the V_H FR1 and V_κ FR4 region, respectively. *Hind*III and *Eco*RI sites are created. The RAFT3ZZ 2-D structure is illustrated in Figure 3.11.

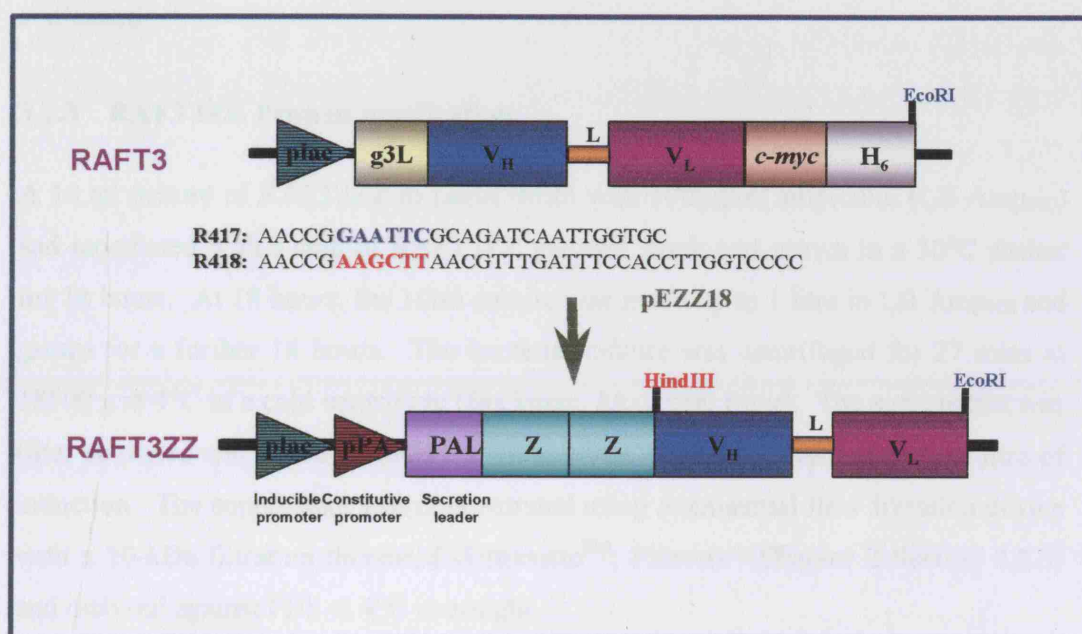


Figure 3.11: Modification of RAFT3 scFv for therapy.

The PCR fragment and vector pEZZ18 were digested with the restriction enzymes *Hind*III and *Eco*RI and then ethanol precipitated. The DNA pellets were washed with 75% ethanol. One hundred ng vector and PCR fragment each were ligated overnight in a water bath set at 15°C. The following morning the ligase was denatured at 68°C and the ligation was butanol precipitated (Chapter 2 Section 2.1.10). The DNA was then transformed into electrocompetent *E. coli* TOP10 bacteria (Invitrogen). TOP10 is deficient in homologous recombination (*recA1*) and was chosen as expression host to

minimise instability of the tandem ZZ repeats. The bacteria were plated on selective agar plates and incubated at 30°C overnight.

The following morning colonies were picked and grown overnight in LB Amp₁₀₀ (Luria Broth (Life Technologies) with 100µg/ml ampicillin) in a shaker at 30°C. The next morning the bacterial culture was centrifuged at 3000 rpm for 5 minutes. The pellets were used to purify the plasmid DNA using a mini prep kit (Promega). The purified DNA was used to carry out diagnostic digests to screen clones for the presence of insert. The supernatant of clones with insert was used to detect melanoma binding of secreted scFv ZZ fusion protein. The intended DNA sequence was then confirmed.

3.2.3 RAFT3ZZ Protein purification

A 10 ml culture of RAFT3ZZ in Luria Broth with 100µg/ml ampicillin (LB Amp₁₀₀) and inoculated with a stab of RAFT3ZZ glycerol stock was grown in a 30°C shaker for 18 hours. At 18 hours, the 10ml culture was made up to 1 litre in LB Amp₁₀₀ and grown for a further 18 hours. The bacterial culture was centrifuged for 27 mins at 18500 g at 4°C in a cold centrifuge (Beckman, JA-10500 rotor). The supernatant was filter sterilised and 1.5ml 100mM PMSF and 5ml 10% NaN₃ were added per litre of induction. The supernatant was concentrated using a tangential flow filtration device with a 10-kDa filtration threshold (UltrasetteTM, Filtron) (Chapter 2 Section 2.2.3) and dialysed against PBS at 4°C overnight.

Two methods of purifying R3ZZ were tested to determine the best method for optimal yield and purity of the protein.

3.2.3.1 Purification on RPL Protein L ColumnsTM

The RAFT3ZZ protein was purified on RPL Protein L ColumnsTM. A Protein L Sepharose[®] 6 Fast Flow column (Amersham Pharmacia Biotech) was washed with 100ml PBS. The concentrated, dialysed and filtered scFv supernatant was loaded onto the column at 0.5 ml/min. An aliquot of the supernatant was retained and the run through was collected and stored at 4°C for further tests. The column was washed with 25ml PBS pH 7.2 to remove unbound protein. The protein was eluted from the

column with 20 ml 0.1M acetic acid pH 2.3. Fractions were collected in 1ml aliquots, neutralised with 250µl 1M Tris base and then assayed by SDS-PAGE and Western Blot under reducing conditions to establish the fractions containing protein and confirm its purity. Fractions containing pure protein were pooled and concentrated to 1ml using an ultra filtration unit concentrator (Vivascience) with a 10 kDa cut off, aliquoted and stored at -80°C. The column was washed with 50ml PBS pH 7.2 plus 0.05% azide and stored at 4°C.

3.2.3.2 Purification on IgG Sepharose ColumnsTM

The RAFT3ZZ protein was purified on human IgG Sepharose columns and eluted with 0.5M acetic acid pH 3.4. An IgG Sepharose[®] 6 Fast Flow column (Amersham Pharmacia Biotech, 10ml bed volume) was equilibrated with two cycles of 30ml 0.5M acetic acid pH 3.4, followed by 30 ml TST buffer (50mM Tris / acetate buffer pH 7.6, 150mM NaCl and 0.05% Tween 20). The concentrated, filtered scFv supernatant was loaded onto the column at 0.5 ml/min. An aliquot of the supernatant was retained and the run through was collected and stored at 4°C for further tests. The column was washed with 5-10 column volumes (50–100 ml) of PBS pH 7.2 to remove unbound protein. Two bed volumes (20ml) of 5mM ammonium acetate (NH₄CH₃COOH) pH 5.0 were applied to the column. The protein was eluted from the column with 20 ml 0.5M acetic acid pH 3.4. Fractions were collected in 1ml aliquots, neutralised with 200µl 1M Tris base and then assayed by SDS-PAGE and Western Blot under reducing conditions to establish the fractions containing protein and confirm its purity. Fractions containing pure protein were pooled and concentrated to 1ml using an ultra filtration unit concentrator (Vivascience) with a 10 kDa cut off, aliquoted and stored at -80°C. ELISA on melanoma cells was used to test melanoma binding by RAFT3ZZ. The column was washed with 100ml TST buffer and 20ml 20% ethanol and stored at 4°C.

3.2.4 Western Blot analysis

Protein samples and gels were prepared as for SDS-PAGE analysis (Chapter 2). Electrophoresis was carried out using the Mini-ProteinTM III system (Biorad) at 200V for 1 hour. Separated proteins were transferred onto Hybond ECL membrane (Amersham International) using a semi dry electroblotting unit (Z34, 050-2, Sigma

Aldrich) [Chapter 2 Section 2.3.3]. Nitrocellulose filters were blocked overnight in 3% skimmed milk. The following morning the nitrocellulose was washed three times with wash buffer (PBS plus 0.05% Tween-20) and incubated for two hours with human IgG (1mg/ml) 1: 1000 in Western blot incubation buffer (PBS, 0.05% Tween-20, 0.1 % BSA). Following the incubation filters were washed as before and then incubated with goat anti human IgG alkaline phosphatase conjugate (Sigma) 1:1000 for two hours. The filters were then washed as before and developed in development buffer and reagents A and B (Biorad). Following sufficient development, the reaction was terminated by washing the filters with tap water.

3.2.5 Gel filtration

The molecular weight of RAFT3ZZ was determined by low-pressure gel filtration on a previously calibrated HiPrepTM Sephacryl-200HR column (Amersham Pharmacia Biotech). Purified RAFT3ZZ (500µg/ml) was loaded onto the column and eluted with PBS. One ml aliquots were collected and tested in ELISA using mouse IgG as primary antibody.

3.2.6 Melanoma binding

A375M cells were seeded in a 96 well flat bottom microtitre plate at 7×10^3 cells per well and grown to confluency (approximately three days in RPMI plus 10% foetal calf serum). The medium was discarded and the plates dried overnight at 37 °C. The bacterial supernatant was titred in the plate and incubated for 2 hours at room temperature with a 1:1000 dilution of purified human IgG (1mg/ml; Sigma) in culture medium plus azide. The plate was washed three times with 200µl PBS per well. Fifty µl of a 1:500 dilution of goat anti-human immunoglobulin horseradish peroxidase conjugate (Dako) was added per well (goat antibodies do not bind protein A and therefore give a lower background). Plates were incubated for one hour and then washed three times with PBS. The plates were stained with 100 µl per well OPD (o-phenylenediamine dihydrochloride; Sigma). The plates were kept in the dark until colour developed. Once stained 50 µl per well 2M sulphuric acid was added to terminate the reaction. A plate reader (Biorad) set at 490nm was used to measure the optical density.

Mouse IgG binding was detected with a 1:1000 dilution of mouse IgG (1mg/ml) and goat anti-mouse immunoglobulin horseradish peroxidase as above.

Melanoma binding was also tested on SK-28 cells. This melanoma cell line expresses the high molecular weight-melanoma associated antigen but is more adherent than A375M cells. Melanoma plates were prepared and melanoma binding by RAFT3ZZ and human or mouse IgG determined as described above.

3.2.7 IgG binding

One μ g RAFT3ZZ was titred two-fold in PBS in a 96 well microtitre plate (Geiner) at 4°C overnight. The following morning the plate was washed three times with 200 μ l PBS-Tween. The plate was blocked for 1 hour at 37°C with 1% bovine serum albumin in PBS and then washed with 200 μ l per well wash buffer (PBS + 0.05% Tween-20). The plate was incubated with a 1:1000 dilution of human IgG (1mg/ml) in PBS for 2 hours. The plate was washed with 200 μ l wash buffer. Fifty μ l of a 1:500 dilution of goat anti-human immunoglobulin horseradish peroxidase conjugate (Dako) in PBS was added per well. The plate was incubated for one hour and then washed three times with wash buffer. The plate was stained with 100 μ l per well OPD (o-phenylenediamine dihydrochloride). The plate was kept in the dark until colour developed. Once stained 50 μ l per well 2M sulphuric acid was added to terminate the reaction. A plate reader (Biorad) set at 490nm was used to measure the optical density.

Mouse IgG binding was detected by using mouse IgG and goat anti-mouse immunoglobulin horseradish peroxidase as above.

3.2.8 C1q binding

Serial dilutions of 250 ng RAFT3ZZ in PBS were made in a 96 well microtitre plate (Geiner) and incubated overnight at 4°C. The following morning the plate was blocked for 1 hour at 37°C with 1% bovine serum albumin in PBS and then washed with 200 μ l wash buffer per well. The plate was then incubated for two hours at room temperature with 50 μ l per well of a 1:1000 dilution of human IgG (1mg/ml). The

plate was washed with 200µl wash buffer and then incubated with C1q 1:1000 (1mg/ml; Sigma) for 2 hours. The plate was washed with 200µl wash buffer per well and 50µl of a 1:500 dilution of goat anti-human C1q horseradish peroxidase conjugate (Dako) in PBS with 0.1% BSA and 0.05% Tween-20 was added per well. The plate was incubated for one hour and washed three times with wash buffer. The plate was stained with 100µl per well OPD (o-phenylenediamine dihydrochloride; Sigma) and kept in the dark until colour developed. Once stained 50µl per well 2M sulphuric acid was added to terminate the reaction. A plate reader (Biorad) set at 490nm was used to measure the optical density.

Mouse IgG binding to RAFT3ZZ and C1q was detected using mouse IgG and goat anti human C1q horseradish peroxidase as above.

3.2.9 Complement Mediated Cytotoxicity

A375M cells are not adherent enough for this assay. Therefore, the strongly adherent human melanoma cell line SK-28 (Carey *et al.*, 1976) was obtained from European Catalogue of Animal Cell Culture (ECACC). SK-28 is a cell line derived from a 51 years old male patient and expresses the high molecular weight melanoma associated antigen (Noronha *et al.*, 1998). Melanoma proteoglycan is trypsin sensitive. Following trypsinisation, 24 hours incubation is required for cells to re-express the antigen on the surface (J. Clarke *et al.*, unpublished). SK-28 cells (1×10^4 cells/well) were seeded in a 96 well plate in RPMI plus 10% foetal calf serum and incubated at 37°C. After 24 hours the medium was aspirated off and 50µl per well serum free medium with RAFT3ZZ at varying concentrations was added in the experimental lanes. Control lanes were incubated with serum free medium only. Human complement serum (Sigma) at varying concentrations was added to all the wells in row A. The complement serum was titred two-fold and the plates were incubated for 24 to 72 hours in a 37°C incubator. The time periods used were selected as optimisation experiments were carried out to achieve maximal CDC. The results of the optimisation experiments are discussed in results section 3.3.8.3.

Following the completion of the incubation period the medium was aspirated off and 100µl per well crystal violet (1.5g crystal violet (BDH), 15ml formal saline, 150ml

ethanol, 2.55g NaCl and 135ml water) was added for 10 minutes. The plate was then washed twice with 200µl per well PBS. The viable melanoma cells remained adherent and took up the stain while all excess dye and non-viable cells were washed away. One hundred µl 33% acetic acid was added per well to lyse the cells. Fifty µl solution was removed from the wells and transferred to a fresh 96 well plate. The optical density was measured in a Biorad plate reader at 595nm. The assay was carried out in triplicates.

Specific complement mediated cytotoxicity was calculated using the equation:

$$\frac{(CS-B)-(Experimental-B)}{(CS-B)} \times 100 = \% \text{ Specific Cytotoxicity}$$

Where CS is complement serum only, experimental is complement serum with RAFT3ZZ and B is background staining by crystal violet stain only. Another negative control carried out but not used in this equation was R3ZZ incubated with SK-28 cells in the absence of human complement serum. Different concentrations of human complement serum, RAFT3ZZ and incubation periods were tested to determine the optimal concentrations for this assay as described in section 3.3.8.

3.2.10 Antibody Dependent Cell Mediated Cytotoxicity

SK-28 cells (1×10^4 cells/well) were seeded in a 96 well plate in RPMI plus 10% FCS and incubated in a 37°C incubator overnight. The following morning the medium was aspirated off and 50µl per well serum free medium with 250ng RAFT3ZZ and human IgG at differing concentrations (see section 3.3.9) was added to the experimental lanes. Control lanes were incubated with serum free medium only. Effector cells (U937- a non-adherent human monocytic cell line; Sundstrom *et al.*, 1976) in serum free medium were added to all the wells in column 1. The effector cells (2×10^6 cells/well) were titred two-fold from column 1 to 12 and the plates incubated for different time periods in a 37°C incubator.

At the end of the incubation period the medium was aspirated and 100µl per well crystal violet was added for 10 minutes. The plate was then washed twice with 200µl

per well PBS. The plate was stained with crystal violet and specific cell lysis calculated using the following formula:

$$\frac{(E-B)-(Experimental-B)}{(E-B)} \times 100 = \% \text{ Specific Cytotoxicity}$$

Where E is effector cells only, experimental is effector cells with RAFT3ZZ and B is background staining by crystal violet stain only. Another negative control carried out but not used in this equation was R3ZZ incubated with SK-28 cells in the absence of effector cells.

Different concentrations of human IgG, time periods, RAFT3ZZ concentrations and effector to target cell ratios were tested to determine optimal conditions for this assay (Section 3.3.9).

3.3 RESULTS

3.3.1 Protein production of RAFT3ZZ

RAFT3ZZ was constructed by cloning the RAFT3 single chain Fv into vector pEZZ18. pEZZ18 is commercially available from Amersham Biotech and was used to clone the single-chain Fv with an N-terminal fusion to 2 ZZ domains (Nilsson *et al.*, 1987). The vector has a protein A promoter and secretion leader, thus resulting in constitutive expression of the protein. In addition, pEZZ18 has the IPTG inducible *lac* promoter of pUC18. The RAFT3ZZ protein was secreted from *E.coli* grown for 18 hours at 30°C. The scFv was purified on IgG Sepharose ColumnsTM. Fractions collected were assayed by SDS PAGE under reducing conditions and stained with Coomassie brilliant blue to establish the fractions containing scFv and confirm its purity. Figure 3.12 shows that the molecular weight of RAFT3ZZ is 39kDa and the fractions typically obtained following purification. Typical RAFT3ZZ purification yield was 500µg protein per litre overnight culture. This is similar to RAFT3 without the ZZ domains. The purified protein was stable at -80°C for over a year. The stability and solubility of RAFT3ZZ were generally improved in comparison with the original RAFT3 scFv.

3.3.2 Protein production of β- Gal ZZ

β- Gal ZZ was purified and stored as described for RAFT3ZZ above. The purification yield obtained for β- Gal ZZ was significantly lower (200µg/l) than for RAFT3ZZ.

3.3.3 Detection of Purified Protein

The purified protein was separated on SDS-PAGE gels and the protein quantified by staining with Coomassie brilliant blue. Figures 3.12 a-d shows results of Coomassie brilliant blue staining of fractions obtained following purification with protein L and IgG Sepharose. Purification with Protein L isolated 2 proteins; one was the size of RAFT3ZZ whereas the second more prominent protein was approximately 27kDa; size of scFv. Purification with IgG sepharose produced pure RAFT3ZZ and was therefore used for all subsequent purifications.

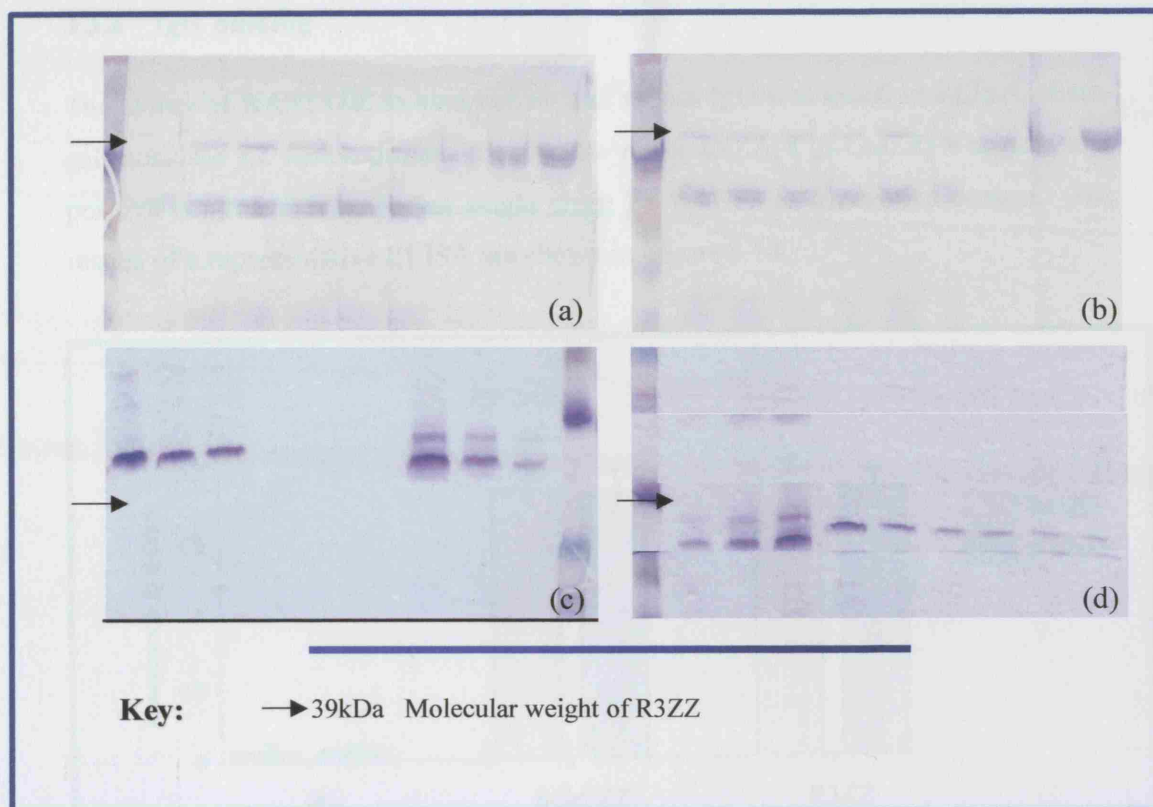


Figure 3.12: Coomassie brilliant blue stained SDS-PAGE of RAFT3ZZ fractions. Gels a (fractions 11-17) and b (fractions 18-23) show purification on a RPL protein L column. Lane 1: Molecular weight marker, lanes 2-7: R3ZZ fractions, lane 8: 1 μ g OVA, lane 9: 3 μ g OVA, lane 10: 5 μ g OVA. Gels c (fractions 7-13) and d (fractions 14-20) show purification by IgG Sepharose. Lane 1: Molecular weight marker, lane 2: 1 μ g OVA, lane 3: 3 μ g OVA, lane 4: 5 μ g OVA, lanes 5-10: R3ZZ fractions.

The protein was tested further and its identity confirmed by Western blot. Figure 3.13 shows a typical Western blot of RAFT3ZZ detected with human IgG.

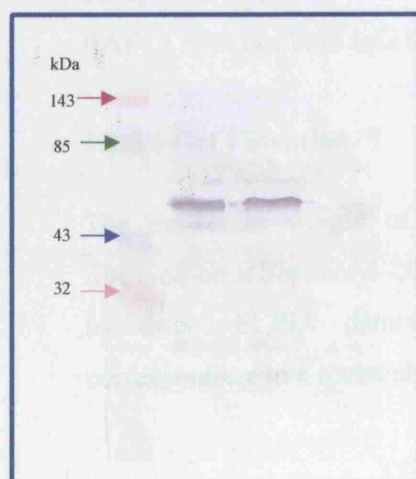


Figure 3.13: R3ZZ staining with human IgG in Western blot. Lane 1: Kaleidoscope marker, lanes 2: fresh prep of R3ZZ, lane 3: a 6 months old prep.

3.3.4 IgG binding

The ability of RAFT3ZZ to bind human and mouse IgG was tested in ELISA. Beta-galactosidase ZZ fusion protein encoded by vector pEZZ18 (β -GalZZ) was used as a positive control and the parent single chain Fv RAFT3 as a negative control. The results of a representative ELISA are shown in Figure 3.14.

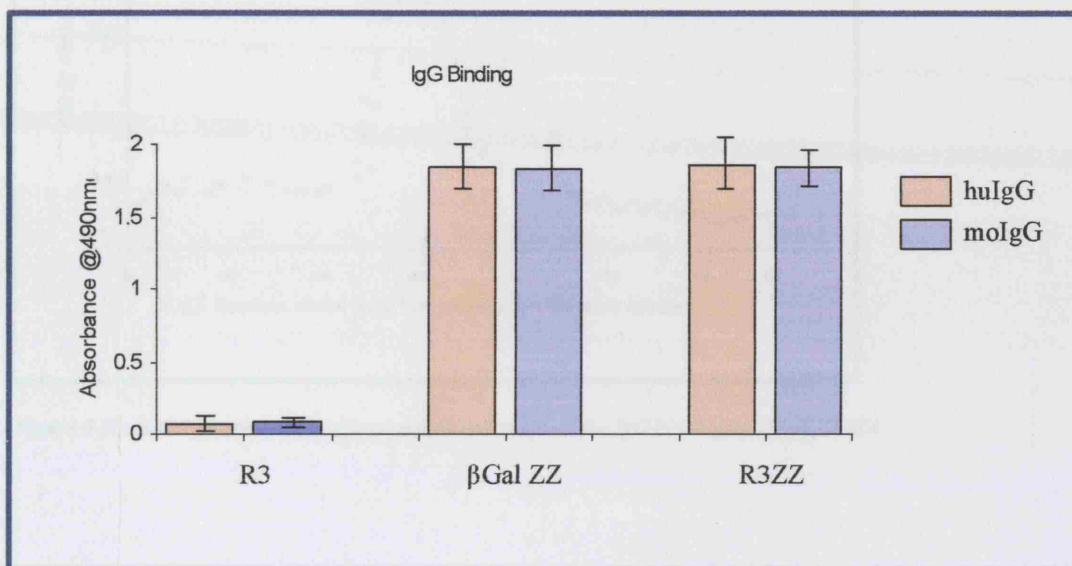


Figure 3.14: Human and mouse IgG binding by the unfused scFv RAFT3, β -Gal ZZ and the RAFT3ZZ fusion protein.

The results show that the RAFT3ZZ (R3ZZ) fusion protein can bind human and mouse IgG as effectively as the β -gal ZZ fusion protein suggesting that the therapeutic antibody could initiate immune activation as effectively as protein A. RAFT3 does not bind IgG thus showing the specificity of IgG interaction with ZZ.

3.3.5 Gel Filtration

The molecular weight of RAFT3ZZ scFv was determined by low-pressure gel filtration on a Sephacryl-200HR column. Detection of RAFT3ZZ in column fractions by direct ELISA demonstrated that RAFT3ZZ emerged as a single peak corresponding to a molecular weight estimated at ~39kDa (Figure 3.15).

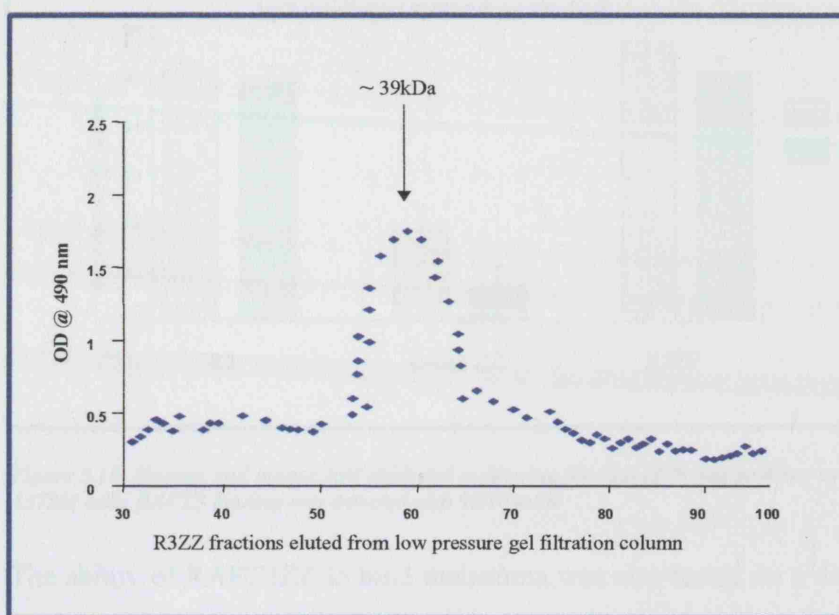


Figure 3.15: R3ZZ fractions eluted from gel filtration tested for IgG binding in direct ELISA.

3.3.6 Melanoma binding

The ability of RAFT3ZZ to bind melanoma was determined by ELISA on A375M melanoma cells. RAFT3 scFv was used as a positive control and the β -GalZZ fusion protein was used as a negative control. The results are shown in Figure 3.16 and demonstrate that RAFT3ZZ binds melanoma as effectively as the RAFT3 scFv. Therefore, the therapeutic construct has retained its melanoma binding ability after modification. The β -GalZZ fusion did not bind melanoma confirming that melanoma binding by RAFT3ZZ is specific. This was also the case when mouse IgG was used to mediate melanoma binding.

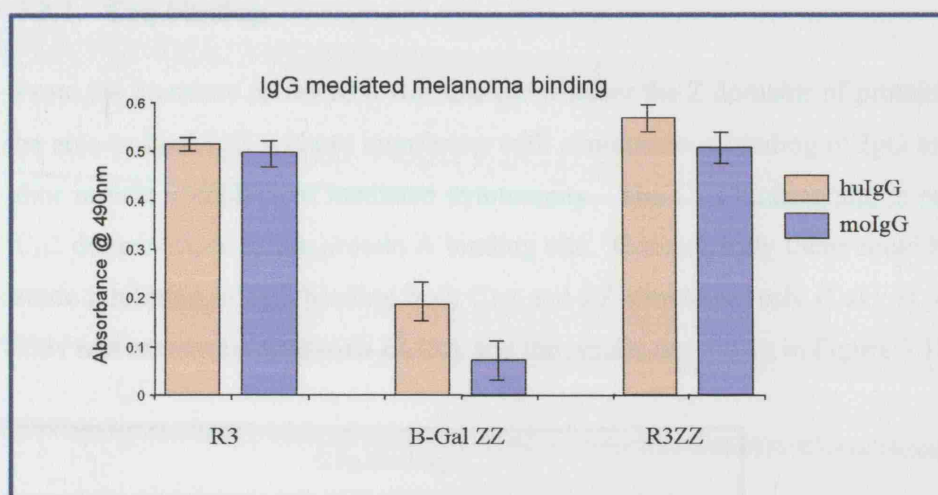


Figure 3.16: Human and mouse IgG mediated melanoma binding of fusion proteins as detected by ELISA on A375M cells. RAFT3 binding was detected with 9E10 mAb.

The ability of RAFT3ZZ to bind melanoma was also tested on a different melanoma cell line expressing the high molecular weight melanoma associated antigen (SK-28). The results (Figure 3.17) show that RAFT3ZZ and RAFT3 both bind the melanoma cells whereas the β -gal ZZ fusion does not bind melanoma confirming that melanoma binding by RAFT3ZZ is specific for the SK-28 cell line.

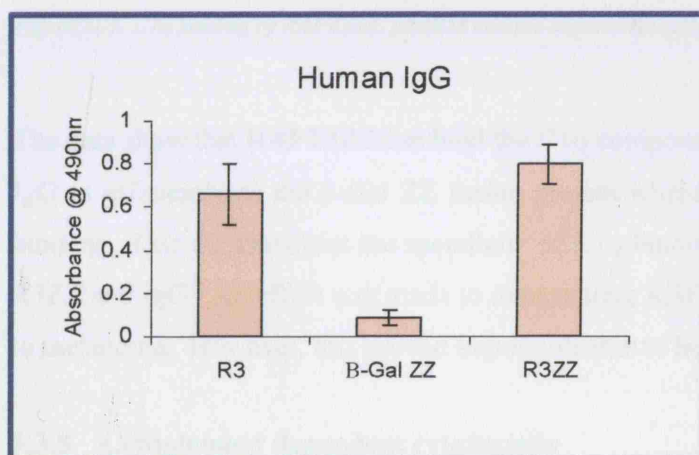


Figure 3.17: SK-28 (melanoma cell line expressing HMW-MAA) binding by RAFT3, RAFT3ZZ and β -Gal ZZ as detected by ELISA with human IgG.

3.3.7 C1q binding

From the literature reviewed it was unclear whether the Z domains of protein A would be able to bind IgG without interfering with simultaneous binding of IgG to C1q and thus initiate complement mediated cytotoxicity. The C1q binding site is on the IgG C_H2 domain close to the protein A binding site. Consequently there could have been steric inhibition of IgG binding both C1q and ZZ simultaneously (Laky *et al.*, 1985). This was tested in a sandwich ELISA and the results are shown in Figure 3.18.

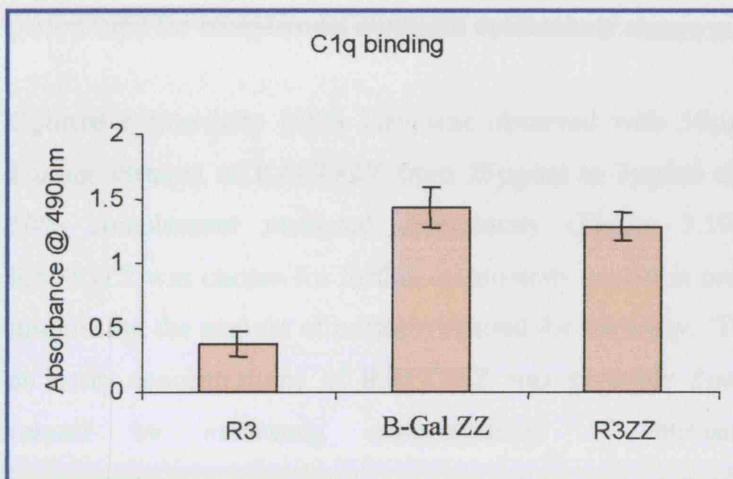


Figure 3.18: C1q binding by RAFT3ZZ, pEZZ18 and the unfused R3 scFv.

The data show that RAFT3ZZ can bind the C1q component of complement via human IgG as efficiently as the β -Gal ZZ fusion protein whereas RAFT3 does not mediate binding. This demonstrates the specificity of C1q binding to melanoma mediated by R3ZZ and IgG. An effort was made to demonstrate RAFT3ZZ mediated C1q binding to melanoma. However, this proved impossible due to high non-specific background.

3.3.8 Complement dependent cytotoxicity

In preliminary experiments we attempted to demonstrate complement mediated melanoma kill by RAFT3ZZ using an LDH release assays. The results showed persistent high background levels (Section 3.1.3.4). Therefore, a crystal violet assay was used to determine the effect of RAFT3ZZ in the presence of human complement serum on the viability of SK-28 melanoma cells. Preliminary experiments were

carried out to optimise the concentration of RAFT3ZZ and human complement serum for this complement dependent cytotoxicity assay.

3.3.8.1 CDC – Optimising Protein Concentration

The first optimisation experiment determined the optimal concentration of RAFT3ZZ for complement-mediated cytotoxicity. RAFT3ZZ (2.5µg/50µl) was titred in 100% human complement serum (Sigma) and the percentage cytotoxicity at 48 hours determined. This time point was used for the assay, as this was the typical incubation period used for complement mediated cytotoxicity assays in the literature.

Optimal cytotoxicity (68% kill) was observed with 50µg/ml RAFT3ZZ per well. Further titration of RAFT3ZZ from 25µg/ml to 3µg/ml also showed approximately 50% complement mediated cytotoxicity (Figure 3.19). Therefore, 25µg/ml RAFT3ZZ was chosen for further cytotoxicity assays in order to maximise kill whilst minimising the amount of protein required for the assay. This is because cytotoxicity at lower concentrations of RAFT3ZZ was probably due to maximal cytotoxicity caused by saturating concentrations of human complement serum.

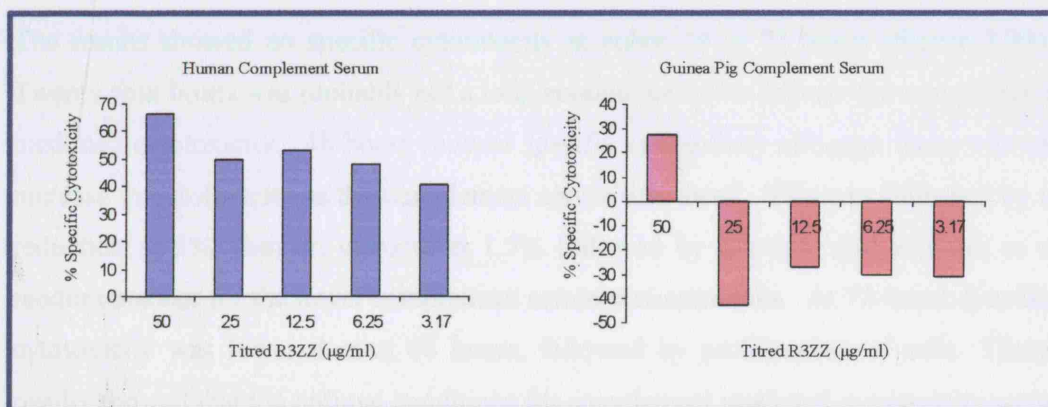


Figure 3.19: The effect of different RAFT3ZZ concentrations on complement mediated cytotoxicity at 48 hours with different complement serum sources as determined by crystal violet viability assay.

3.3.8.2 CDC – Source of Complement Serum

Human complement serum was costly. Therefore, an alternative source of complement serum was also tested. Guinea pig complement was less expensive and was therefore tested as an alternative source of complement proteins. However, from the results in Figure 3.19 it can be seen that guinea pig complement serum was not effective in mediating specific cytotoxicity. Instead, proliferation of cells was observed. Therefore, guinea pig complement was not pursued further as an effective alternative complement source. It was therefore decided that human complement serum should not be substituted for an alternative source of complement serum, as this would also make extrapolation of the results to humans more difficult.

3.3.8.3 CDC – Optimising Incubation Periods

The next factors to be optimised were the incubation period and concentration of human complement serum. All experimental wells contained 12.5µg/ml RAFT3ZZ. Fifty percent human complement serum was titred in normal medium used for cell culture. Time periods of 24, 48 and 72 hours were tested as it covered the majority of time periods used by a number of studies.

The results showed no specific cytotoxicity at either 24 or 72 hours (Figure 3.20). Twenty four hours was probably not a long enough period to activate the complement mediated cytotoxicity. 48 hours showed specific cytotoxicity although there was an increase in cytotoxicity as the complement serum was titred. This was followed by a reduction at 3%, then an increase at 1.5% followed by decrease and increase in a random manner for the lower complement serum concentrations. At 72 hours specific cytotoxicity was lower than at 48 hours, followed by proliferation of cells. These results showed that the optimal conditions for complement mediated cytotoxicity were 48 hours with 12.5µg/ml RAFT3ZZ.

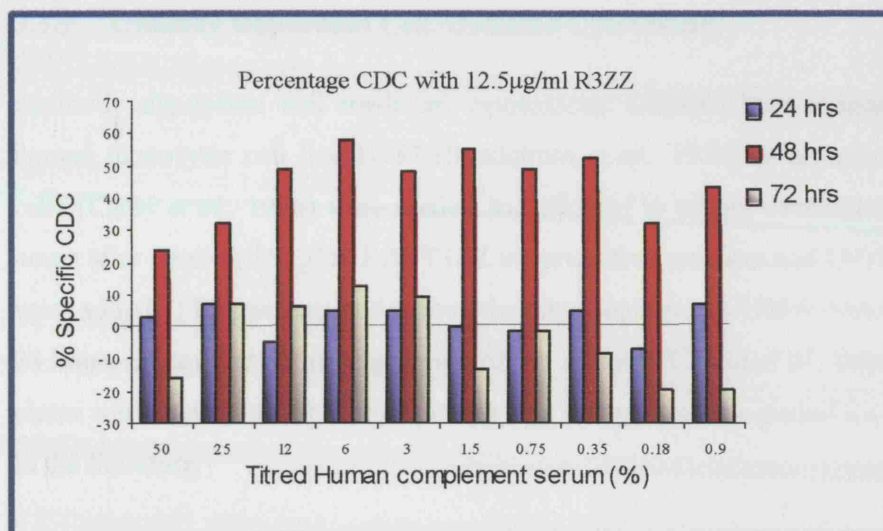


Figure 3.20: The effect of different incubation periods on CDC at varying human complement serum concentrations.

Finally, different human complement serum concentrations were tested. The optimal conditions established above were used and human complement serum was titred.

The CDC assay was repeated under the conditions found to be optimal as a result of Figure 3.20 and 65 % maximal CDC was observed after 48 hours with 12.5µg/ml R3ZZ and 50% human complement serum. The results of complement-mediated cytotoxicity under these conditions are shown in Figure 3.21. Therefore, incubation of SK-28 cells with 12.5µg/ml RAFT3ZZ and 50% human complement serum were established as optimal for this CDC assay.

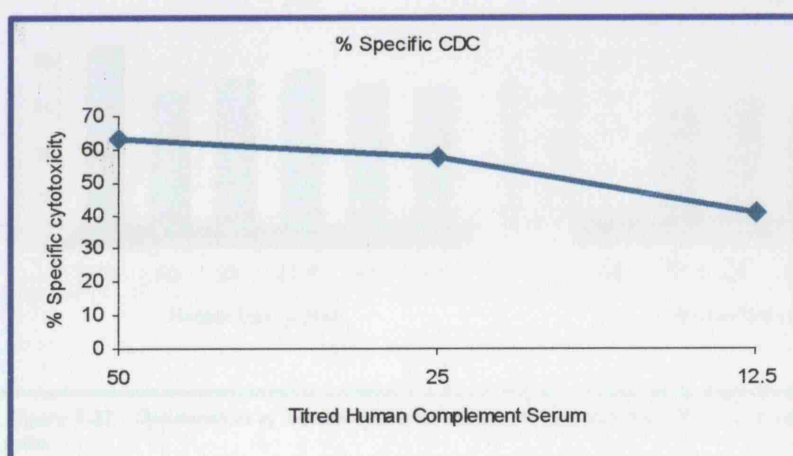


Figure 3.21: Optimal specific complement mediated cytotoxicity with RAFT3ZZ.

3.3.9 Antibody Dependent Cell Mediated Cytotoxicity

Antibody dependent cell mediated cytotoxicity (ADCC) was measured using the human monocytic cell line U937 (Sundstrom *et al.*, 1976) as effector cells. SK-28 cells (Carey *et al.*, 1976) were seeded and allowed to adhere overnight. Twenty four hours after seeding 25µg/ml RAFT3ZZ in serum free medium and U937 effector cells were added. [Trypsinisation destroys the target epitope on HMW-MAA. A period of 24 hours is required for re-expression of the antigen (Clarke *et al.*, unpublished). The plates were incubated for 4 hours as this was the typical time period for ADCC assays in the literature.

The concentrations of human IgG, RAFT3ZZ and the effector to target cell ratios were optimised. Initially the human IgG concentration used for ADCC was optimised. In the initial experiment with RAFT3ZZ at 12.5µg/ml and an effector to target cell ratio of 200 was used. Human IgG was titred from 100µg/ml to 3.6µg/ml. The results showed an optimal kill by ADCC of 80% at a concentration of 100µg/ml (Figure 3.22a). However, even at lower concentrations there was no significant difference between the percentage ADCC observed. Thus it was concluded that the effector cells were at saturating levels and therefore not a good indicator for determining the optimal IgG concentration. The experiment was therefore repeated with an effector to target ratio of 100 (Figure 3.22b).

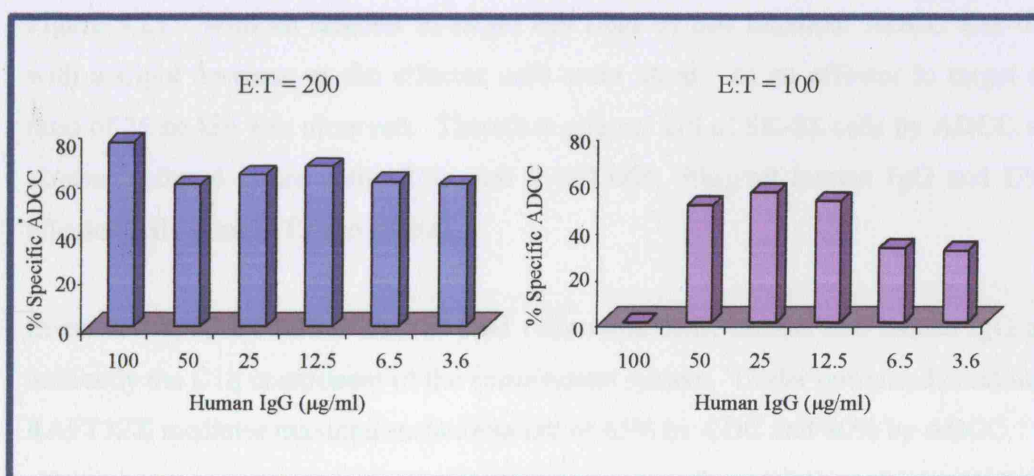


Figure 3.22: Optimisation of human IgG concentrations required for ADCC at 4 hours. E:T, effector to target ratio.

The results showed that at an effector to target cell ratio 100:1 there is no significant difference in percentage ADCC at IgG concentrations of 50µg/ml to 12.5µg/ml. At 100 µg/ml no ADCC was observed but this was probably an anomalous result as the previous experiments had shown specific ADCC at this concentration. Therefore, 50µg/ml was assumed as optimal IgG concentration and was used at a fixed concentration for subsequent experiments.

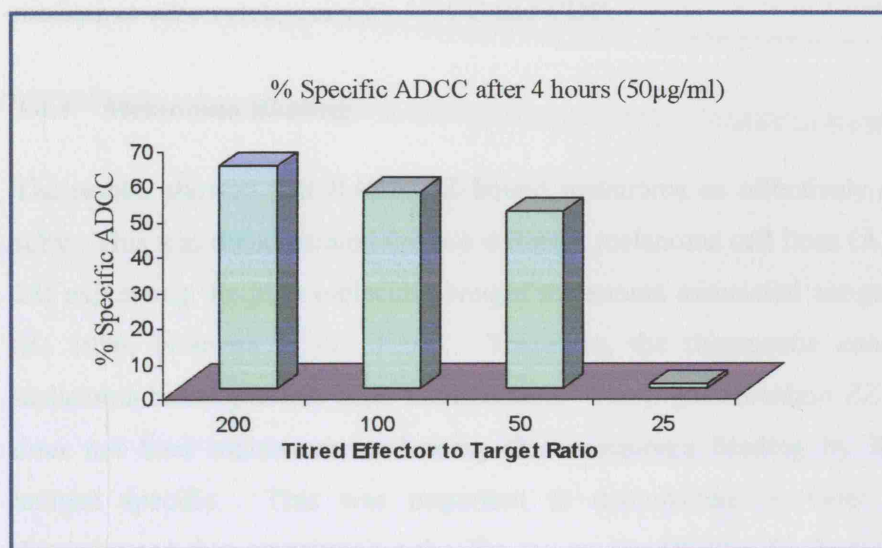


Figure 3.23: Percentage specific ADCC after 4 hours with titrated effector to target cell ratio. ADCC – Optimising Effector to Target Cell Ratio.

Finally, the effect of effector to target ratio was tested and the results are shown in Figure 3.23. With an effector to target cell ratio of 200 maximal ADCC was 65% with a slight decrease as the effector cells were titred. At an effector to target cell ratio of 25 no kill was observed. Therefore optimal kill of SK-28 cells by ADCC was obtained after 4 hours with 12.5µg/ml RAFT3ZZ, 50µg/ml human IgG and U937 effector cells at an E:T ratio of 200.

In summary, RAFT3ZZ is able to bind melanoma cells, human and mouse IgG and indirectly the C1q component of the complement system. Under optimised conditions RAFT3ZZ mediates maximal melanoma kill of 65% by CDC and 80% by ADCC.

3.4 DISCUSSION

The aim of the work described in this chapter was to use RAFT3ZZ to redirect polyclonal IgG of most isotypes to melanoma.

The results demonstrate that the therapeutic construct RAFT3ZZ is able to bind human and mouse IgG, the C1q component of the complement system (via IgG) and mediate *in vitro* cytotoxicity by ADCC and CDC.

3.4.1 Melanoma Binding

The results showed that RAFT3ZZ bound melanoma as effectively as the RAFT3 scFv. This was demonstrated for two different melanoma cell lines (A375M and SK-28) expressing the high molecular weight melanoma associated antigen (Pluschke *et al.*, 1996; Noronha *et al.*, 1998). Therefore, the therapeutic construct retained melanoma binding ability after modification. The β galactosidase ZZ fusion protein does not bind melanoma confirming that melanoma binding by RAFT3ZZ was antigen specific. This was important to demonstrate as Goel *et al.* (2000) demonstrated that repositioning the His-tag on the CC49 scFv from the C-terminal position to the N-terminus adversely affected the antigen binding properties of the protein. The authors highlighted the importance of functional characterisation of recombinant proteins expressed with differing purification tags. The melanoma binding results confirmed that the N-terminal position of the ZZ domains of the recombinant protein does not appear to interfere sterically with epitope recognition by RAFT3ZZ. Furthermore, the expression yields of RAFT3 and RAFT3ZZ were similar.

3.4.2 C1q Binding

From the literature reviewed it was unclear whether the Z domains derived from protein A would be able to bind C1q via IgG and thus initiate CDC. This was because the C1q binding site is on the IgG C_H2 domain close to the protein A binding site (Laky *et al.*, 1985). Laky *et al.* (1985) demonstrated competition of the B domain of protein A with C1q for IgG binding and suggested the possibility of steric inhibition of IgG binding to both C1q and protein A simultaneously via the two C_H2

domains of the IgG molecule. However, inhibition was temperature dependant (at 4°C but not at 37°C) and this was not observed with RAFT3ZZ. Our ELISA that was carried out at room temperature only shows some C1q binding to IgG bound to the RAFT3ZZ coated plate. The difference observed between the two studies could be due to the pentameric protein A (42kDa) used by Laky *et al.* 1985 preventing binding of C1q (460kDa) to IgG indirectly because of its size whereas the binding of the smaller ZZ domain (14kDa) to C_H2 does not preclude binding of C1q. If this was the case, then reconstruction of RAFT3ZZ diabodies could possibly be more effective than generation of RAFT3 fusions to whole protein A (see below). It is possible that some steric hindrance exists *in vitro* if R3ZZ is used in excess of IgG (as both binding sites are bound by R3ZZ) but this should not occur *in vivo*, as IgG would be in excess of FART3ZZ and C1q.

All *in vitro* experiments were carried out with human and mouse IgG. The *in vivo* model is a mouse model and the therapeutic effect of RAFT3ZZ in mice should be similar to that in humans allowing a relatively realistic extrapolation to humans. This is important as at present *in vivo* models especially for the active immunotherapy of melanoma remain unsatisfactory. As a result mouse data can be difficult to extrapolate to humans. This will be discussed in detail in Chapter 5.

3.4.3 Cytotoxicity Assays

Having demonstrated that RAFT3ZZ binds to all the necessary components required for antibody dependent cell mediated and complement mediated cytotoxicity the percentage cytotoxicity was determined. This presented a bigger challenge than was first anticipated. This was largely due to the high background readings caused by the human complement serum and the effector cells required. Background is a common problem with serum analysis by ELISA on melanoma cells (Kupsch *et al.*, 1999). Pure C1q was not available.

Cytotoxicity assays that were considered included single cell mediated cytotoxicity, the ⁵¹Cr release assay, the ³H proline release assay, the live/dead cytotoxicity assay (Molecular Probes), the CytoTox 96® Non Radioactive cytotoxicity assay (Promega) and finally the crystal violet assay.

3.4.3.1 Chromium Release Assay

The chromium release assay is the most commonly used assay to measure ADCC (<http://www.lef.org/protocols/prtcl-027f.shtml>). It was originally described by Thorn *et al.* (1974) who used effector cells that were activated by appropriate cytokines or non-specifically. Target cells are labelled with Na⁵¹CrO₄, washed and then incubated with effector cells at different ratios, typically for 4 hours. Triton X-100 is then added to the target cells and release of total radioactivity measured using a gamma counter. The disadvantage of using the chromium release assay is the need of radioactivity and the cost required to establish and then optimise ADCC and CDC assays. From a practical point of view radioactive assays incur high costs of disposal. Furthermore, radioactivity decays relatively quickly. Should there be problems with the growth of effector or target cells non-radioactive assays are preferable.

3.4.3.2 ³H Proline Release Assay

The ³H proline release assay (Saal *et al.*, 1976) is similar to the ⁵¹Cr release assay and therefore poses the same advantages and disadvantages. Furthermore, the disposal of ³H is an environmental issue that raises laboratory costs. Tritium has a half-life of 12.4 years and waste cannot be stored for decay. The typical costs for disposal of radioactive waste is £200 per mCi. In spite of this radioactive assays remain popular for measuring ADCC and CDC. In part this is due to the high background levels in non-radioactive CDC assays using complement serum.

3.4.3.3 Flow Cytometry Assay

Liu *et al.* (2002) developed a non-radioactive flow-cytometry assay to measure CTL-mediated killing of target cells and antigen-specific cellular immune responses. It also enables quantification of the target-cell killing activities mediated by cytotoxic T lymphocytes (CTLs). This assay was deemed to be unsuitable for our study due to the errors associated with manual counting of cells and the possibility of introducing bias in the results.

3.4.3.4 CytoTox 96® Non Radioactive Assay

Initial cytotoxicity assays were performed using the CytoTox 96® Non Radioactive Cytotoxicity Assay (Promega). This assay is a colorimetric alternative to the ^{51}Cr release cytotoxicity assay widely used in the literature. The CytoTox 96® Assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis in the same manner that ^{51}Cr is released in radioactive assays. Ito *et al.* (1996) have shown excellent correlation of the results obtained with this assay and the standard chromium release assay.

This assay was at first deemed a suitable alternative to the radioactive assays. However, during the course of the study some limitations of this assay became apparent and it was discontinued. The limitations included the high background levels observed and the cost. High background levels could, according to the manufactures, be due to the source of serum or the phenol red in culture medium. Phenol red free medium was tested but this did not resolve the problem. As our assay required complement serum to mediate CDC it was not possible to reduce the serum concentrations in the assay. Thus it was deemed necessary to find an alternative assay.

3.4.3.5 Crystal Violet Assay

The crystal violet assay requires adherent tumour cells. The advantage of this assay is that the number of viable cells can be determined by measuring the optical density (Ishiyama, *et al.*, 1996). The background problems associated with complement serum are limited in this assay as all supernatant is removed and thus only the effect on cell vitality is measured.

Following consideration of the assays that were available it was decided that the crystal violet assay would be the most suitable for our study. This assay is more commonly used to measure proliferation of target cells. The target cells must be adherent allowing the non-viable cells (i.e. cells in the medium) to be washed off in the washing steps therefore leaving only live cells adhered to the plate. The percentage specific cytotoxicity is then determined.

3.4.4 Optimisation of CDC and ADCC

Various factors were taken into consideration in an attempt to optimise the cytotoxicity assays. These included incubation period, concentration of the protein, concentration of human complement serum, effector to target cell ratio and finally the number of target cells to be used.

Guinea pig complement serum was found to be an unsuitable source of complement serum. These findings were similar to those of Idusogie *et al.* (2000) who showed there are species-specific differences in complement activation with rituximab (a mouse/human chimeric Mab). The authors also observed that Rituximab appeared to be less effective in conferring lysis when used with guinea pig complement than with human or rabbit complement.

Following optimisation of CDC, 65% tumour specific cytotoxicity was obtained after 48 hours with 12.5µg/ml RAFT3ZZ. The percentage cytotoxicity observed was comparable to 50% observed by Shopes *et al.* (1992) using a similar protein concentration. However, the RAFT3ZZ concentration was high compared to Holliger *et al.* (1997) who used 10ng/ml diabody to lyse erythrocytes coated with target antigen. This could be a reflection of the relatively high complement resistance of melanoma cells.

Maximal ADCC with 12.5µg/ml RAFT3ZZ, 50µg/ml human IgG and U937 effector cells at an E:T ratio of 200 was obtained after 4 hours. The percentage cytotoxicity observed was comparable to that previously reported by others. Sung *et al.* (1995) examined ADCC by chimeric antibodies using natural killer cells as effectors. Their typical results were 62% ADCC with 1µg/ml antibody after 4 hours. Table 3.3 shows other examples of *in vitro* melanoma cytotoxicity by a range of antibodies.

Mab	Isotype	Antigen	CDC	ADCC	Study
Km641	MoIgG ₃	GD3	32.4% (5µg/ml)	41.8% (5µg/ml) huPBMCs (100:1)	Ohta, 1993
E26-1	HuIgG ₁	HMW	96.7% (1µg/ml)	93.5% (1µg/ml) hu NK cells (20:1)	Wang, 1999
9.2.27	MoIgG _{2a}	HMW	ND	16% (10µg/ml) hu PBMCs (100:1)	Ortaldo, 1987
225.28s	MoIgG _{2a}	HMW	0%	33.2% (4µg/ml) mo splenocytes (800:1)	Imai, 1982
653.40s	MoIgG ₁	HMW	0%	20.4% (4µg/ml) mo splenocytes (800:1)	Imai, 1982
7c11.e8	HuIgM	GD3/GM3	72%	ND	Abdel-Wahab, 1993
KM871	HuIgG ₁	GD3	45% (5µg/ml)	45% (5µg/ml) huPBMCs (100:1)	Shitara, 1993

Table 3.3: Examples of melanoma kill in vitro by both ADCC and CDC.

The kill assays could be optimised further by the use of IL-2 to prime the effector cells prior to incubation with the target cells. Ortaldo *et al.* (1987) have shown enhanced melanoma kill by ADCC using human NK cells by priming effector cells with IL-2, IFN- γ and IFN- β . Cytokines such as GM-CSF, IL-2, IFN- α and IFN- β , TNF- α and TNF- β have been shown to activate macrophages (Belardelli *et al.*, 1995) and may enhance melanoma kill.

Holliger *et al.* (1997) used specific diabodies and showed that antibodies retargeting serum IgG to monocytes in combination with T cell retargeting can exhibit a strong synergistic effect. The possibility that ADCC and CDC may have a higher combined cytotoxicity than that observed in individual assays could also be tested in future experiments. For example, one could incubate melanoma cells for 44 hours with RAFT3ZZ and complement to mediate CDC and then add the monocytes to mediate ADCC for the last 4 hours.

A synergy between T cells and NK cells (Fc γ IIIR positive) has also been described *in vitro* and *in vivo* (Kipriyanov *et al.*, 2002). Synergy is therefore one possibility that may be explored further.

3.4.5 Growth Inhibition vs Cytotoxicity

In principle, it is possible that RAFT3ZZ could halt proliferation of melanoma cells rather than mediating specific cytotoxicity. However, the SK-28 cells used for this assay divide once every 24 hours. Therefore, although the observed percentage CDC could be explained by growth inhibition, the high percentage of ADCC at 4 hours excludes this possibility. Secondly, one of the negative controls for CDC was the incubation of SK-28 cells without human complement serum in the presence of RAFT3ZZ. This was used as a negative control to demonstrate specific cytotoxicity therefore ruling out the possibility that RAFT3ZZ in itself could halt proliferation of melanoma cells.

The crystal violet assay does not differentiate between melanoma kill and mere cell growth inhibition. Eighty five percent reduction in the ADCC assay after 4 hours is most certainly melanoma kill, however 65% reduction in CDC could be attributed to growth inhibition rather than melanoma kill. The susceptibility of SK-28 cells to kill by ADCC and CDC was previously shown by Ohta *et al.* (1993) who observed 32.4% CDC and 41.8% ADCC with KM641 monoclonal antibody at 5µg/ml (Table 3.3).

The primary effect of complement should be lysis, however, cells can recover from complement attack. MAC becomes clustered and then either endocytosed or expelled as membrane residues into the culture supernatant (Morgan, 1999; Marchbank *et al.*, 1997). This could in part explain the lack of CDC at 72 hours (Figure 3.20) due to recovery of SK-28 cells. However, SK-28 cells in the absence of both human complement serum and RAFT3ZZ were included in some assays. These showed results similar to those obtained by incubation with serum only (used as negative control to calculate percentage specific cytotoxicity) and RAFT3ZZ.

C1q binds the assembled C5b-C8 complex in the membrane leading to a conformational change in C9. More C9 molecules join the assembly to form a ring of C9 molecules known as the membrane attack complex (MAC). CD59 inhibits the assembly of C9 into the MAC. CD59 and similar complement inhibitory molecules (CD35, CD46 and CD55) are expressed in abundance by melanoma (Weichenthal *et al.*, 1999). It is therefore possible that kill of melanoma by R3ZZ is effective *in vitro*

but not *in vivo*, as CD59 is the major factor protecting melanoma against complement kill (Brasoveanu *et al.*, 1995; 1996; Maio *et al.*, 1998). In principle the *in vivo* role of complement could be tested in complement deficient mice (Di Gaetano *et al.*, 2003)

Another possibility would be the use of peripheral blood mononuclear cells (PBMCs) as a source of effector cells as these include diverse effector cells such as natural killer cells, macrophages and granulocytes. This possibility was tested initially. However, due to the large number of cells that were required for each experiment and for optimisation it was not feasible to obtain sufficient quantities of blood from colleagues. Therefore, an established monocytic cell line was used as an initial external source of effector cells. This could be investigated further to compare the effect of a monocytic cell line compared with PBMCs. The use of RAFT3ZZ together with bispecific anti T- cell anti melanoma scFv currently under development in our laboratory would also be of interest.

3.4.6 Future Work

Despite the demonstration of successful melanoma kill by the construct *in vitro*, attempts were made to improve RAFT3ZZ by further engineering. These attempts included cloning different numbers of protein A domains and cloning into a better vector to improve bacterial growth and expression yield. The therapeutic molecule used in this study consists of a scFv fused to two artificial domains of protein A. By modifying the construct with differing numbers of Z domains it would be possible to analyse how this affects therapy. It is known that *in vivo* complement activation is achieved more readily with IgM molecules than with IgG molecules due to its pentameric IgM structure (Kuby *et al.*, 2000). It should also be noted that IgM is more potent than IgG in CDC but does not mediate ADCC. Caron *et al.* (1992) and Shopes (1992) have made dimeric IgG with a C-terminal disulphide bond. In both cases CDC was increased *in vitro*. Only Caron *et al.* (1992) tested the dimers in ADCC and found an increase in tumour kill. Therefore, the use of dimers is another possibility that could be explored in the future.

Therefore, the possibility that a five-domain structure would be more effective at initiating CDC or ADCC could be investigated. It may be that with an increased

number of protein A domains the therapeutic construct will have an increased ability to recruit IgG molecules *in vivo* and thus result in a more effective therapy. However, the increased number of protein A domains should result in an increased size of complexes with IgG. Such molecules might therefore lose the advantage of good tumour penetration, could lead to steric inhibition of C1q binding (Laky *et al.*, 1985) and thus may not improve therapy. For example, the binding of 5 IgG molecules by a scFv fusion molecule to natural protein A could lead to melanoma – independent Fc receptor cross linking and non-specific activation of NK cells and monocytes. An increased number of protein A domains could therefore also result in increased side effects.

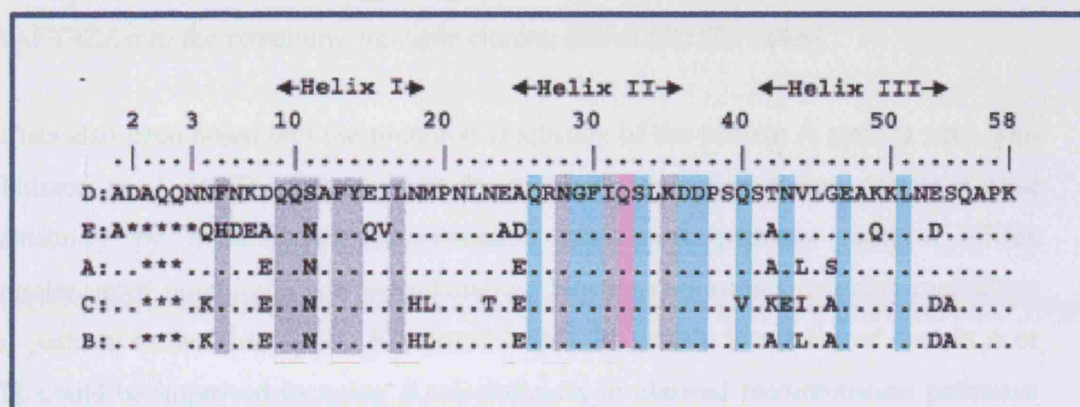


Figure 3.24: The differences between different protein A domains.

Similarly it is possible that reducing the number of Z domains to 1 could improve the kill or reduce side effects. RAFT3ZZ has 2 Z domains and could in principle cross link high affinity FcR in an antigen dependent manner. Ligation of R3Z with a low affinity FcR would require antigen binding for activation e.g. of neutrophils. Although no non-specific side effects were observed in the present study (Chapter 5) these remain to be investigated when using increased doses and other treatment schedules.

Therefore, attempts were made to construct therapeutic molecules with one artificial Z domain or 1-5 domains of protein A (domains E, D, A, B and C). The cloning strategy was to amplify different domains of protein A by PCR and then fuse them to the RAFT3 scFv in pUC119his. Despite a number of attempts no protein-expressing

clones were obtained. Another problem was that it was virtually impossible to design domain-specific primers that would not cross prime amplification of homologous gene segments of protein A. Due to time limitations this approach was abandoned.

These problems could be overcome by the use of alternative techniques such as gene synthesis instead of PCR (Nilsson *et al.*, 1987) or more suitable cloning hosts e.g. other *E. coli* strains. The fusion of whole protein A to RAFT3 posed a different problem. It is possible that vector pRIT2T (Nilsson *et al.*, 1985) is unstable since the vector is no longer commercially available from Amersham Biotech. A clone with a transcription factor cloned in this vector was obtained from ATCC. When the bacteria were streaked out and grown to produce a fresh glycerol stock the plasmid had lost the cloned insert suggesting instability of the vector. Attempts to clone RAFT3ZZ into the remaining multiple cloning site of pRIT2T failed.

It has also been noted that the mutation frequency of the protein A gene is very high (Nilsson, *et al.*, 1987). This could be due to selective pressure against protein A gene instability by homologous recombination (the *recA* pathway requires ≥ 50 bp uninterrupted homology) for recombination. However, plasmids can also recombine by pathways other than *recA*. It is possible that the genetic instability of protein A or ZZ could be improved by using *E. coli* deficient in plasmid recombination pathways (e.g. *E. coli* Sure, Stratagene).

Whilst carrying out work with RAFT3ZZ we encountered problems with the stability of this construct. The growth properties of the bacterial culture varied from one preparation to another. This could be due to the constitutive expression of RAFT3ZZ in the pEZZ18 vector and resulting bacterial toxicity. In an attempt to make this construct more stable it was cloned into vector pBAD gene III. This is a commercially available vector (Invitrogen) based on the pBAD vector series of Guzman *et al.* (1995). The pBAD/gene III plasmids are pUC derived expression vectors designed for tightly regulated, secreted recombinant protein expression and purification in *E. coli*. The gene III signal sequence is utilised for secretion of the recombinant protein into the periplasmic space. For example, pBAD arabinose has been used for the expression of scFv GFP fusion protein that could not be expressed under the control of the less tightly controlled *lac* promoter (DeLisa *et al.*, 1999).

Therefore, the optimal conditions for high levels of secreted, recombinant protein expression have to be determined empirically.

Attempts to express RAFT3ZZ in this vector were also unsuccessful. This was due to the low expression in the *E. coli* strain LMG194. *E. coli* Top10 is *rec A* deficient whereas LMG194 is *rec*⁺ (Guzman *et al.*, 1995). This strain allows repression for low basal level expression of toxic genes by D-glucose and induction with L-arabinose. After repeated attempts to optimise expression of RAFT3ZZ in pBAD gene III the construct was produced at low levels and did not show convincing melanoma binding despite of the correct insert sequence. This may be because the induction and thus expression of the clones requires further optimisation. The two Z gene segments could form recombinants with 1, 2 or more Z sequences in this strain. Nilsson *et al.* (1987) used *rec*⁻ strains to produce vectors with 2 (pEZZ18), 5 and 10 Z domains. The authors found that 5 and 10 Z domains were unstable in *rec*⁺ strains. The mini Z domain is still >50bp but the mini ZZ version could show less homologous recombination than the whole protein A gene. If recombination was the problem, then another future direction could be to create a ZZ vector with ZZ domains that differ by silent mutations in the wobble base (third position) of codons, i.e. disrupting the minial 50bp stretch of homology required for *recA* mediated recombination. Nilsson *et al.* (1987) noted a high mutation frequency of whole protein A in this position and suggested that there could be a selective pressure for the evolution of such genetically stabilising mutations. Finally, hybrid protein A/ Protein G have been made with a different isotype binding profile that could be fused to scFvs as a means of fine tuning them for a better therapeutic potency (Kato *et al.*, 1995).

Peptides of 10 –20 amino acids have been identified as short but high affinity protein binders. However, Braisted *et al.* (1996) have demonstrated that it is also possible to make a functional protein domain substantially smaller whilst retaining the functional activity of the protein. For example, the Z domain (53 residues) has an IgG binding affinity of $K_d=10\text{nM}$ and its two helix derivative (33 residues) has a binding affinity of $K_d=43\text{nM}$. This study demonstrated that smaller alternatives to protein A binding domains are possible. Although this alternative was not available at the time of this study it is a possibility for the future direction of the work.

At the time when this project commenced RAFT3 was the best characterised scFv available. However, during the course of this study other scFvs have been developed which may prove to be more efficient therapeutic scFvs due to higher apparent stability of the proteins *in vivo*. These include scFvs that form dimers or multimers. Some of these scFvs have yields of up to 10-fold higher than RAFT3 and RAFT3ZZ. Therefore, another method of increasing the cytotoxic effect of RAFT3ZZ could be to use scFv diabodies, tribodies or multimers as fusions with ZZ as this would result in the recruitment of more IgG molecules and thus may increase the recruitment of killer cells *in vivo*. For example, a RAFT3 scFv diabody ZZ fusion molecule should have 4 IgG binding sites compared with the 2 IgG binding sites of RAFT3ZZ. This could result in increased complement kill, prolonged serum residence but may also reduce tumour penetration.

3.4.6.1 Experimental Update

Attempts have been made by my successor to improve scFv ZZ expression by subcloning the scFv collection made by phage display (rounds 3, 4 and 5 separately) in bulk into pEZZ18. Phage display is a selection technique in which a peptide or protein is expressed as a fusion with a coat protein of a bacteriophage resulting in display of the fused protein on the exterior surface of the phage virion. The plasmid DNA encoding the fusion resides within the virion. Phage display has been used to create a physical linkage between vast libraries of random peptide sequences to the DNA encoding each sequence, allowing rapid identification of peptide ligands for a range of target molecules (antibodies, enzymes, cell-surface receptors, etc.) by an *in vitro* selection process called “panning” (Parmley *et al.*, 1988; Noren, 1996). Panning is carried out by incubating a library of phage-displayed peptides with a plate (or bead) coated with the target, washing away the unbound phage and eluting the specifically-bound phage. (Alternatively the antibody phage can be reacted with the target antigen in solution, followed by affinity capture of the phage-target complexes onto a plate or bead that specifically binds the target.) The eluted phage is then amplified and taken through additional cycles of panning and amplification to successively enrich the pool of phage in favor of the tightest binding antibodies. After 3–4 rounds, individual clones are characterized by DNA sequencing and ELISA. Phage display offers a powerful and general method to change and refine the

properties of a protein or peptide that can be displayed on the phage surface (Swanson, 1999).

The clones were selected for good expression and melanoma binding. RAFT3 and 3A11 or similar clones were the most frequently isolated ZZ fusion clones. There seemed to be a selection against spontaneous multimers, perhaps due to daisy chain formation through weak V_H binding by ZZ (but kinetically favoured due to juxtaposition). This would lead to the formation of large non-covalent scFv ZZ complexes that cause translational jamming and expression toxicity (Hayhurst, 2000). Therefore, R3ZZ is indeed a good clone in pEZZ18.

Chapter 4

Immunogenicity

4 IMMUNOGENICITY

4.1 INTRODUCTION

4.1.1 Monoclonal Antibodies in Cancer Therapy

From the early days of monoclonal antibody discovery until today there has been a significant effort to develop antibodies for cancer therapy. The use of unmodified monoclonal antibodies has a number of limitations (Tangri *et al.*, 2002). Problems that hinder the ultimate utility of these reagents include the immunogenicity of monoclonal antibodies (Khazeli *et al.* 1994), slow elimination from the blood (Colcher *et al.*, 1998) poor vascular permeability, low and heterogeneous tumour uptake (Kortt *et al.*, 2001), cross-reactivity with normal tissues and metabolism of immunoconjugates (Reilly *et al.*, 1995). The clinical experience with 2 well-characterised antibodies, Rituximab and 17-1A, will be described in detail.

4.1.1.1 Rituximab

Rituximab (anti-CD20) antibody is an example of a therapeutic monoclonal antibody engineered to reduce the HAMA response in humans. Rituximab is a chimeric Mab that consists of human IgG₁ and kappa constant regions and variable regions from the murine IgG₁ Mab IDEC-2B8. It targets CD20 with high affinity in patients with Non-Hodgkin's lymphoma. Maloney *et al.* (1997) observed 50% complete and partial responses with mild infusional toxicity (375mg/m² four times a week for four weeks). Symptoms with this high dose treatment schedule included fever, chills, rash and nausea. However, the response rate was not as high in the subsequent Phase 2 study by Coiffier and co-workers. Fifty-four patients were included in the trial of which 5 gave a complete response and 12 patients showed a partial response (Coiffier *et al.*, 1998). Rituximab was then tested in combination with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) chemotherapy for untreated low grade Non-Hodgkin's lymphoma. The combination of monoclonal antibody therapy with chemotherapy achieved a complete response in 22 of 38 patients. The remaining 16 patients had a partial response with no additive toxicity to CHOP when compared with CHOP alone (Czuczman *et al.*, 1999).

For patients with advanced-stage intermediate-grade B-cell lymphoma, an interim analysis of a phase 3 trial performed by the Groupe d'Etude des Lymphomes de L'Adulte (GELA trial LNH 98-5) recently indicated a significantly improved complete response rate (76% vs 63%), event-free survival (69% vs 49%) and 1-year survival (83% vs 68%) in 328 elderly patients (60-80 years of age) treated with Rituximab plus CHOP compared with CHOP alone (Magni *et al.*, 2000; Vose *et al.*, 2001; Coiffier *et al.*, 2002). The authors concluded that the addition of Rituximab to the CHOP regimen increases the complete-response rate and prolongs event-free and overall survival in elderly patients, without exhibiting clinically significant increase in toxicity (Coiffier *et al.*, 2002).

Although Rituximab has been shown to be successful in combination with chemotherapy for Non-Hodgkin's lymphoma until recently trials using monoclonal antibodies for solid tumours have shown disappointing results (Green *et al.*, 2000). Herceptin (Trastuzumab) for the treatment of breast carcinoma overexpressing the EGF receptor family HER-2 antigen (Leget *et al.* 1998) and 17-1A (Edrecolomab) in preventing relapse in patients with Duke's C resected colon cancer (Riethmuller *et al.* 1994) are promising examples of monoclonal antibody therapy for solid tumours.

4.1.1.2 17-1A Monoclonal Antibody

Monoclonal 17-1A antibody therapy has been extensively tested in clinical trials since the early 1980s (Sears *et al.*, 1982). First objective anti tumour responses in patients were rare, less than 5-10% (Sears *et al.*, 1984). A major factor contributing to the poor response rate was the large quantity of antibody (doses greater than 400mg) required to achieve significant binding of the antibody to the tumour (LoBuglio *et al.*, 1988). The side effects were generally mild and included diarrhoea, nausea, vomiting, abdominal pain and fever. However, upon repeated administration most patients developed a human anti-mouse antibody (HAMA) response (Sears *et al.*, 1984).

Further studies with the 17-1A antibody tested immune mediated tumour lysis with 17-1A mAb. Velders *et al.* (1994) constructed a second generation mouse / human

chimeric antibody (MAb 323/A3) derived from 17-1A. It was speculated that chimerisation should reduce the immunogenicity and increase interaction with human effector mechanisms. The authors observed higher levels of ADCC and CDC with chimeric 323/A3 than with 17-1A. Furthermore, the chimeric 323/A3 was equally active as chimeric 17-1A at 100 to 1000-fold lower concentrations (Velders *et al.*, 1994). Studies by LoBuglio *et al.* (1989) showed that mouse/human 17-1A chimeric antibody had a six-fold longer circulation with substantially lower immunogenicity than the mAb. Meredith *et al.* (1991) confirmed these findings with ¹³¹I labelled chimeric mouse/human antibody in colon cancer patients.

The immunogenicity is a specific property of the antibody used. Mouse/human chimeric Mab B72.3 induces immune responses in 50-70% of patients (Meredith *et al.*, 1992; Khazaeli *et al.*, 1991). These were mainly anti-idiotypic responses.

4.1.2 Genetically Engineered monoclonal Antibodies

The disappointing results from clinical trials with mouse mAbs promoted the use of genetically modified monoclonal antibodies (Khazaeli *et al.*, 1994). Genetic engineering of monoclonal antibodies has the potential to optimise their therapeutic efficacy whilst reducing side effects. Genetic engineering was believed to provide the answers for many of the problems associated with monoclonal antibodies. These included reducing the HAMA response, enhancing effector functions, altering the pharmacokinetics, increasing penetration into tumour masses, increasing affinity and developing more efficient delivery of drugs, toxins or biologic modifiers (Maloney *et al.*, 2000).

One of the reasons for generating scFv fragments was to produce a fragment that would reduce the HAMA response and alter the pharmacokinetics of blood clearance. It was shown that by reducing the size of the whole monoclonal antibody the smaller size antibody fragment exhibits faster clearance from the body (Colcher *et al.*, 1990; Begent *et al.*, 1996). Furthermore, it was thought that antibody fragments retaining antigen binding but with a smaller size (Fab, Fv) should reduce the immunogenicity of the antibody (Kuus-Reichel *et al.*, 1994).

4.1.3 Immunogenicity of scFvs

The potential of scFv fragments to trigger an anti-idiotypic immune response was studied by Reinartz *et al.* in 2000. In order to improve the anti-idiotypic response in patients the authors generated scFv fragment ACA125. This was derived from the parent anti-idiotypic mAb that was able to initiate an anti-idiotypic response that cross reacted with the tumour antigen. Anti-id antibody titres were lower than triggered by immunisation with the complete mAb and an increase of specificity was not observed. The rat anti-(mouse Ig) antibody (RAMA) responses detected in rats post immunisation were stronger than in rats immunised with parent mAb ACA125 (Reinartz *et al.*, 2000). This shows that both the idiotypic and non-idiotypic parts of mouse V domains are immunogenic in rats. This contradicts the expectation that human antibodies, chimeric antibodies and mouse Fab fragments should be much less likely to induce anti-antibody responses than intact mouse monoclonal antibodies or mouse F(ab)₂ fragments, when only a single injection is administered in patients (Kuus-Reichel *et al.*, 1994).

4.1.4 Therapeutic Use Of ScFvs

It is a general belief that single chain Fv antibody fragments are very weakly immunogenic. Single chain Fv antibodies and their derivatives have been used for therapy with a number of strategies. Various attempts have been made to make these antibody fragments immunogenic.

4.1.4.1 ScFv -Tetanus Toxin Fusion

Stevenson *et al.* (1995) showed that using a myeloma derived V_H and V_L in scFv format low levels of anti-idiotypic antibody and poor protective immunity are observed in mice. To provide an alert signal to the immune system and to generate high levels of T cell help, King *et al.* (1998) fused tetanus toxin fragment C to the scFv. Vaccination with the fusion protein induced protection against challenge with 5T33 myeloma compared with non-vaccinated mice whereas the unmodified parent scFv was ineffective.

4.1.5 Immunogenicity of Therapeutic Proteins in Humans

Fierlbeck *et al.* (1994) showed that natural human IFN β is more immunogenic than human recombinant IFN β . Another example of a recombinant protein that is less immunogenic than the natural human protein is IFN α 2b (Kontsek *et al.*, 1999). Human GM-CSF made in yeast and *E. coli* is immunogenic due to the lack of glycosylation (Gribben *et al.*, 1990). Humanised Trastuzumab has been found to be non-immunogenic in patients with metastatic breast cancer (Baselga *et al.*, 1999). However, Matteson *et al.* (1995) showed that humanised CAMPATH-1 Mab is immunogenic in rheumatoid arthritis patients. Mab MEDI-493 humanised anti-IL-2 receptor, and the human Mab Daclizumab (humanised Mab against IL-2 receptor used to treat transplant rejection and auto-immune disease) have also been shown to be immunogenic in patients (Brekke *et al.*, 2003).

4.1.6 Anti-idiotypic Responses to ScFv

The anti-idiotypic response varies from antibody to antibody. A good anti-idiotypic response requires an Fc part for efficient interaction of antibody with antigen presenting cells (Durrant *et al.*, 2000; Tao and Levy, 1993). It may also require a T_H neo-epitope formed by V(D)J joining during the secondary immune response. Attempts to stimulate an anti-idiotypic response with scFv without Fc portion have been made by incorporating scFvs into liposomes. Zeytin *et al.* (2000) used an anti-idiotypic scFv as a DNA vaccine against sarcoma and small cell carcinoma. However, multiple immunisations were required to elicit strong immune responses.

McCormick *et al.* (2003) used human lymphoma derived scFvs to induce an anti-idiotypic response in mice that did not cross react with other scFvs. The idiotype scFvs were expressed and could be used in plants for treatment of Non-Hodgkin's Lymphoma. Human lymphoma derived scFvs fused to a immunoenhancing sequence from tetanus toxin were also constructed and shown to induce an anti-idiotypic response in mice without cross reactivity to other human scFvs (Forconi *et al.*, 2002). Caspar (1997) generated an anti-idiotypic against human scFv in mice. However, the response was weak and fusion of scFv to GM-CSF did not enhance the response. Furthermore, King *et al.* (1998) only observed an anti-idiotypic response in mice when a mouse scFv was fused to tetanus toxin C.

Magliani *et al.* (1998) made a mouse Ab2 scFv that mimics *candida albicans* killer toxin. The immunogenicity was not tested specifically but the Ab2 scFv protected rats from *candida albicans* infection. The authors then made a mouse anti-idiotypic Ab that mimicked a streptococcal antigen. Immunisation of mice induced Ab3 reactive with *Streptococcus* and protected mice from infection (Magliani *et al.*, 1998).

Pavalinkova *et al.* (2001) tested mouse, human/mouse, and human/human (humanised) chimeric scFvs for cross reactivity with sera from patients that were treated repeatedly with the mouse parent monoclonal antibody. The human scFv showed no cross-reactivity with patient sera. The authors concluded that human scFvs should be non-immunogenic without immunising mice or humans with scFvs. However, in view of the afore-mentioned studies this assumption may be false.

4.1.7 Epitopes

The therapeutic potential of an antibody depends on its ability to recruit the immune response after binding to a disease-specific epitope. A B cell epitope is a site on an antigen that is recognised by an antibody or a surface IgG receptor on B lymphocytes. Proteins, haptens and rarely lipids and carbohydrates can also form T cell epitopes. A T-cell epitope usually is a short continuous peptide derived from a protein antigen. It binds to an MHC molecule and is recognised by a particular T cell clone. B-cell epitopes are antigenic determinants recognised by B cells. They are typically discontinuous in the primary peptide structure or they can be carbohydrates or haptens. Neo-epitopes are artificial epitopes that can be created as a result of antigen preparation. E.g., enzymatic cleavage of collagen or expression in heterologous systems such as insect cells can create nepepitopes (Lark *et al.*, 1997). In nature, neoepitopes can also be formed by mutation of TAAs, VDJ junction during antibody maturation or disease processes.

4.1.7.1 Continuous Epitopes

In linear (continuous) binding sites the key amino acids mediating the contacts with the antibody are located within one part of the primary structure that is usually not greater than 15 amino acids in length. Peptides covering these sequences can have

affinities for antibodies that are within the range of the intact protein ligand (Figure 4.1a).

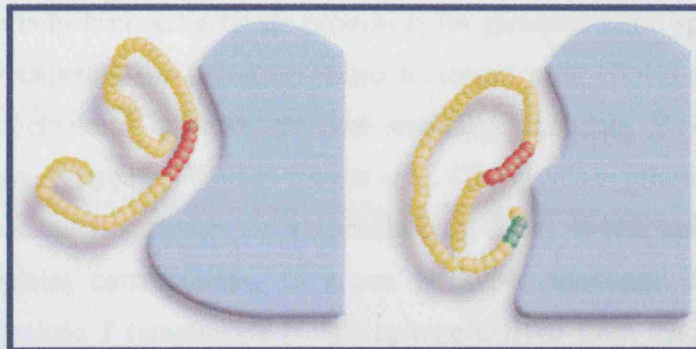


Figure 4.1(a) Continuous epitope (left) and (b) discontinuous epitope (right). In linear (continuous) epitopes the key binding residues are located within one stretch of the antigen's primary structure (left) whereas conformational (discontinuous) epitopes are composed of two or more binding regions which are separated in the primary but adjacent in the tertiary structure (right). Adapted from <http://www.jerini.de/base.htm>.

4.1.7.2 Discontinuous Epitopes

In conformational (discontinuous) binding sites the key residues are distributed over two or more binding regions, which are separated in the primary structure. Upon folding these binding regions are brought together on the protein surface to form a composite epitope. Even if the complete epitope mediates a high affinity interaction, peptides covering only one binding region, as synthesised in a linear scan of overlapping peptides, generally have very low affinities that often cannot be measured in normal ELISA or Biacore experiments (Figure 4.1b).

4.1.7.3 B Cell Epitopes

The ability to function as a B cell epitope is determined by the nature of the antigen binding site on the antibody molecules displayed by B cells (Kuby *et al.*, 2000). The antibody binds to an epitope by van der Waals (weak, non covalent) bonds and recognises the surface pattern, usually consisting of the amino acid pattern of charged polar, hydrophobic or bulky side chains on a protein. B cell epitopes are accessible and on the exposed surface of the immunogen. They generally contain hydrophilic amino acids and are often located at surface exposed loops that connect elements of secondary structure (α -helix or β -strands) or where there is a high degree of segmental

mobility (atomic mobility). B cell epitopes may be continuous or discontinuous [section 4.17.1]. The antigen-binding site of the antibody molecule determines the size of the B cell epitope. The size of B cell epitopes is in the range of 650 to 855 Å² (Davies, 1996) for proteins. It is 274 Å² for the hapten digoxin (Jeffrey *et al.*, 1993).

Carbohydrate antigens are promising for generation of a specific antitumour response by targeting the immune system to cancer cells (Fredman *et al.*, 2003). However, carbohydrate epitopes do not normally stimulate T cell responses. This was demonstrated by Vichier-Guerre *et al.* (2003) who synthesised a glycopeptide vaccine containing a cluster of a carbohydrate TAA B-cell epitope covalently linked to peptides corresponding to a pan HLA-DR 'universal' T-helper epitope and to a cytotoxic T lymphocyte (CTL) epitope derived from the carcino embryonic antigen (CEA). A strong T-cell dependent antibody response specific for the Tn antigen, in both out bred and HLA transgenic mice was observed. Therefore, the pan-T_H cell epitope stimulates a functional anti-carbohydrate B cell response that is independent of the MHC class II haplotype.

4.1.7.4 T Cell Epitopes

T cells recognise small fragments of proteins (peptides) bound to polymorphic products of the major histocompatibility complex (MHC) on cells (Townsend *et al.*, 1987). Peptides of 8-10 amino acids are presented by class I molecules bound to a cleft on the MHC class I heavy chain and recognised by CD8⁺ T cells. These antigenic peptides are usually derived from cleavage of intracellular proteins. Similarly, MHC class II molecules bind 8-25 amino acid peptides, generally derived from the cell membrane or extracellular proteins and are predominantly recognised by CD4⁺ T-lymphocytes (Braciale *et al.*, 1991). Abnormal antigenic peptide processing, transport and presentation by MHC class I and II can lead to escape of melanoma cells from immune surveillance (Fossati *et al.*, 1986). This is described in more detail in Chapter 3.

4.1.8 MHC Class I Pathway

Nucleated cells express class I MHC. Proteins are fragmented in the cytosol by proteasomes (a complex of proteins with proteolytic activity) or other proteases and transported across the endoplasmic reticulum membrane by transporter proteins. Genes within the MHC complex encode the transporter proteins and some components of the proteasome. Synthesis and assembly of class I heavy chain and β_2 microglobulin then takes place in the endoplasmic reticulum resulting in the formation of a stable complex. The complex is transported to the cell surface and presented to $CD8^+$ cytotoxic T cells (Williams *et al.*, 1996). The process of MHC class I antigen presentation is illustrated in Figure 4.2.

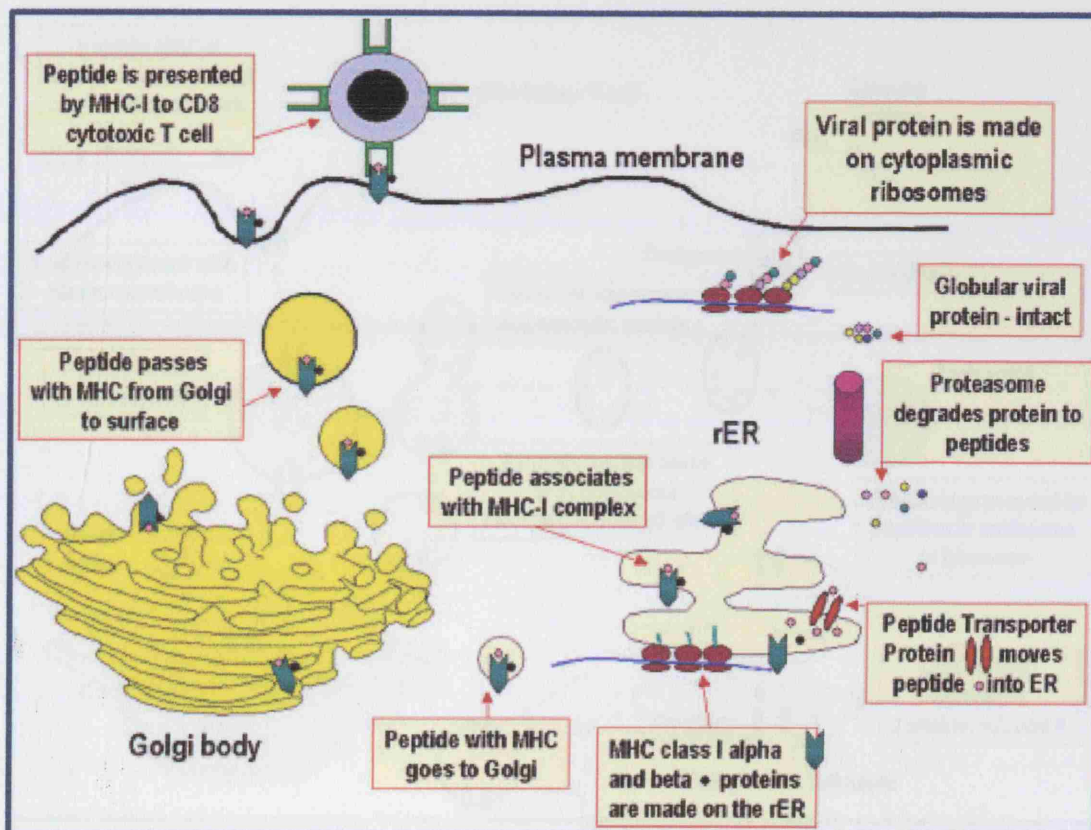


Figure 4.2: Pathway of MHC class I restricted presentation of an endogenously synthesised antigenic peptide. An example of such an antigen would be a viral protein made in the cell as a result of infection (<http://www.med.sc.edu:85/bowers/ant-pres.htm>).

4.1.9 MHC Class II Pathway

A limited group of cells (mainly macrophages, dendritic cells (Langerhans cells) and B cells) express MHC class II. Exogenous proteins are taken up by endocytosis and fragmented by proteases in the lysosomal or endosomal compartment. The alpha and beta chains of MHC class II, and the invariant chain are synthesised, assembled in the endoplasmic reticulum and transported through the Golgi apparatus to the endosomal compartment. The invariant chain is then digested and the peptide fragments from the exogenous protein associate with the class II MHC molecules and are transported to the cell surface (Weenink *et al.*, 1997). This process is illustrated in Figure 4.3. The peptide MHC II complex is presented to CD4⁺ helper T cells.

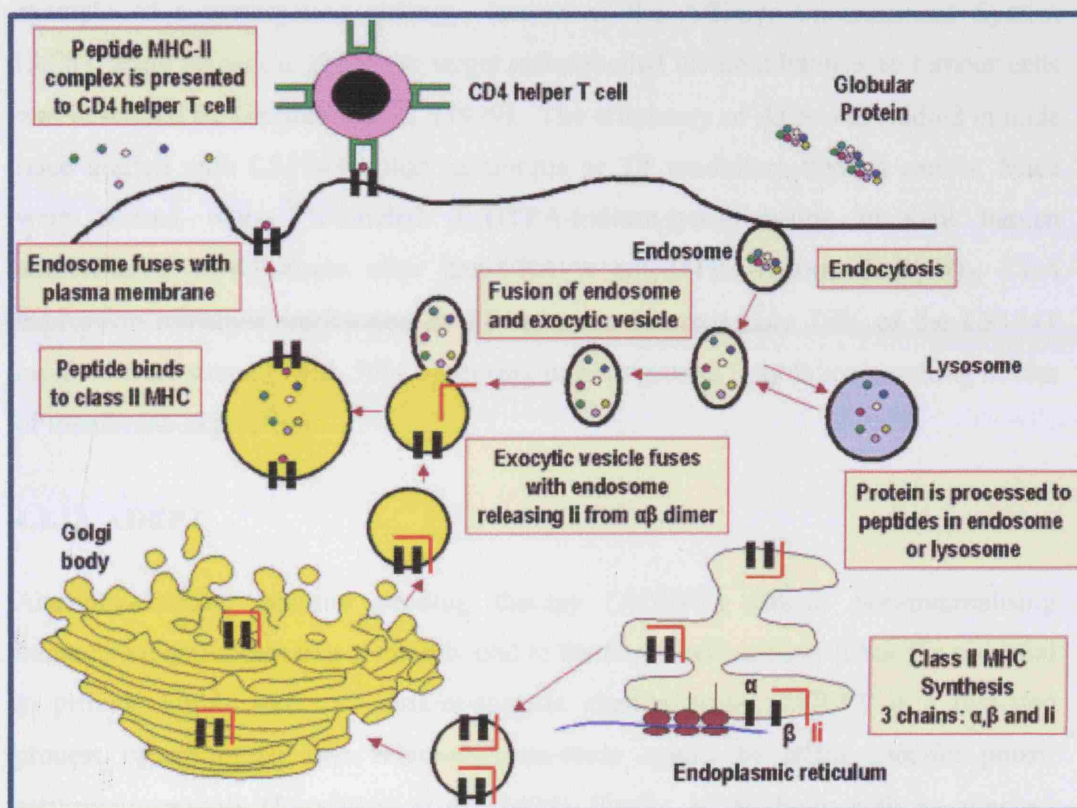


Figure 4.3: Pathway of class II MHC-restricted presentation of an exogenous antigen (<http://www.med.sc.edu.85/bowers/ant-pres.htm>).

4.1.10 Haptens

A hapten is a small molecule that cannot induce an immune response by itself. The hapten can bind to the paratope on an antibody. Haptens can induce an antibody response if they are conjugated to larger carrier molecules (normally proteins). If conjugated to a carrier protein, T_H cells will recognise epitopes of this protein and B cells will recognise the hapten. This results in the production of anti hapten antibodies as well as other antibodies directed at the carrier molecule.

4.1.11 Pretargeting antibodies with haptens

Haptens have been used for diagnostic and therapeutic tumour pretargeting. An example of a pretargeting strategy, known as the Affinity Enhancement System (AES), using bispecific F(ab')₂ to target radiolabelled bivalent haptens to tumour cells was described by Gautherot *et al.* (1999). The efficiency of AES was studied in nude mice grafted with LS174T colon carcinoma or TT medullary thyroid cancer. Mice were treated with ¹³¹I-labeled di-DTPA-indium-tyrosyl-lysine bivalent hapten administered 15-48 hours after anti-CEA x anti-DTPA-indium BsF(ab')₂. CEA expression remained unchanged in TT tumours, whereas only 70% of the LS174T tumours were stained, with 58% observing tumour growth retardation resulting in loss of membrane expression.

4.1.12 ADEPT

Antibody-directed enzyme prodrug therapy (ADEPT) utilises non-internalising monoclonal antibodies that remain bound to the target cell surface. It has the potential to provide highly selective tumour-specific chemotherapy. ADEPT is a two-step process incorporating two relatively non-toxic agents to attain specific potent antitumour activity (Bagshawe *et al.*, 1999). Firstly, an antibody with an attached enzyme component is administered and allowed to localise specifically on the surface of tumour cells, thereby targeting enzyme molecules to the surface of cancer cells. In the second step, inactive forms of anti-cancer drugs (prodrugs) are administered and converted to their fully active cytotoxic form by the enzymes that have been localised to the tumour. This results in enzymatic conversion of prodrug to drug specifically at

the site of the tumour. The advantages of ADEPT therapy are reduced exposure of normal tissue to the drug whilst maximising concentrations in tumour tissue.

4.1.13 Melanoma Epitopes

Epitope expression, MHC expression, an appropriate T cell repertoire and B cell epitopes are all believed to be necessary for effective immunotherapy of melanoma patients (Maeurer *et al.*, 1996).

Antigen	HLA	Epitope	Sequence
Testis-specific antigens			
MAGE-1	A1	161-169	EADPTGHSY
	Cw16	230-238	SAYGEPRKL
MAGE-3	A1	168-176	EVDPIGHLY
	A2	271-279	FLWGPRALV
	B4	167-176	MEVDPIGHLY
	A2	195-203	IMPKAGLLI
BAGE	Cw16	2-10	AARAVFLAL
GAGE-1	Cw6	9-16	YRPRRRY
PRAME	A24	301-309	LYVDSLFFL
Melanocyte differentiation antigens			
gp100	A2	154-162	KTWGQYWQV
	A2	209-217	ITDQVPFSV
	A2	280-288	YLEPGPVT A
	A2	457-466	LLDGTATLRL
	A2	476-485	VLYRYGSFSV
	A3	17-25	ALLAVGATK
MART-1	A2	27-35	AAGIGLTV
	A2	32-40	ILTVILGVL
Tyrosinase	A2	1-9	MLLAVLYCL
	A2	369-377	YMNGTMSQV
			YMDGTMSQV
	A24	206-214	AFLPWHLRF
	B44	192-200	SEIWRDIDF
TRP-1/gp75	A31	1-9	MSLQRQFLR
TRP-2	A31	197-205	LLPGGRPYR
Mutated and aberrantly expressed antigens			
MUM-1	B44	782-808	EEKLIVVLF
b-catenin	A24	29-37	SYLDSGIHF
P15	A24	9-18	AYGLDFYIL
CDK4	A2	23-32	ACDPHSGHFV
GnT-Ve)	A2		VLPDVFIRC V
Gp100-in4	A24	170-178	VYFFLPDHL

Figure 4.4. Antigenic epitopes of melanoma associated antigens recognised by CTL (adapted from Zeuthen *et al.*, 1998).

Most melanoma associated epitopes have been identified by CTL clones. Examples of T cell epitopes identified on melanoma antigens and their HLA allele presentation are shown in Figure 4.4. The identification of these melanoma- specific epitopes provides avenues for the immunotherapy of melanoma patients. Wang *et al.* (1999c) described one such study using a MART-1 peptide vaccine in a phase I trial of resected high-risk melanoma patients. Patients with high-risk resected stages IIB, III, and IV melanoma were immunised with a vaccine comprised of an immunodominant peptide derived from the MART-1 tumour antigen with incomplete Freund's adjuvant. The authors observed 40-50% patients developed an immune response to peptide-pulsed targets or tumour cells. Furthermore, the immune response correlated with prolonged relapse-free survival.

4.1.14 Mapping an Epitope

A number of methods are available for epitope mapping. Some of the most commonly used techniques are discussed below.

4.1.14.1 Pepscan Assay

The Pepscan assay is used to define B and T cell epitopes and makes use of synthetic peptides but can only be used if the amino acid sequence of the antigen is known. It can be adapted for the delineation of epitopes recognised by both helper T cells and cytotoxic T cells. The identification of combined B and T cell epitopes by using synthetic peptides have also been reported.

Goetsch *et al.* (2003) immunised mice with the protein G derivative BB, performed B and T pepscan analyses and compared the results with pepscan of human sera and T cells. This identified T helper cell epitopes, linear B cell epitopes and one conformational B cell epitope in BALB/c mice. One T_H epitope was shared by mice and humans. Other examples where this method has been used to map epitopes includes Cerino *et al.* (1993); Williams *et al.* (1998); Mason *et al.* (1999) and Lundkvist *et al.* (2002) to map B cell epitopes.

4.1.14.2 Site Directed Mutagenesis

Site directed mutagenesis is also a powerful tool in epitope mapping and can be used to evaluate the role of individual amino acids in immune complex formation. Examples of studies using site directed mutagenesis to confirm epitope location include Boehm *et al.* (2000) investigating the carcinoembryonic antigen (CEA); Smethurst *et al.* (2004); Spangfort *et al.* (2003) studying the dominating IgE-binding epitope of Bet v1, the major allergen of birch pollen and Wang *et al.* (2003) identifying four sites of stimulated tyrosine phosphorylation in the MUC1 cytoplasmic tail. Michael *et al.* (1996) used site directed mutagenesis to introduce unique cloning restriction sites into CPG2 gene. This enabled production of biologically active recombinant fusion proteins that could be expressed in *E. coli* and possess enzyme activity with anti -CEA specificity for ADEPT.

Another use of site directed mutagenesis is for mapping binding sites. This method was utilised by Idusogie *et al.* (2000) for mapping the C1q binding site on a chimeric antibody. The authors constructed point mutations at specific sites and tested the mutant antibodies for C1q binding, thus mapping the C1q binding site to the D270, K322, P329 and P331 positions on human IgG₁.

4.1.14.3 General Methods For Epitope Mapping

Other monoclonal antibody assays such as enzyme linked immunosorbent assay (Hufert *et al.* 1989; Reineke *et al.* 1999; Renukaradhya *et al.* 2002), haemagglutination (Deslauriers *et al.* 1992) and the immunoreactivity pattern in Western blots (Renukaradhya *et al.* 2002) may also be used for epitope mapping. Random peptide phage display libraries can be selected with antibodies and the cloned peptide amino acid sequence used to identify the epitope (Scott and Smith 1990). However, peptide-based assays are inadequate when identifying discontinuous epitopes (Klein and Horejsi 1997).

The use of mass spectrometry for the analysis of proteins has triggered the development of new strategies for the identification of discontinuous epitopes.

Limited proteolysis cleaves the antigen by different proteases in the presence and absence of the antibody. The antibody fragments are then identified by mass spectrometry. The region of the antigen protected from proteolysis upon binding of the antibody contains the epitope (Suckau *et al.*, 1990).

Selective chemical modification is the reaction of the antigen with chemical tags specific for Arg, Tyr, and Lys residues. The sites of modification in the free and antibody-bound antigen are determined by mass-spectrometric peptide mapping (Fiedler *et al.*, 1998). In epitope excision antibody coated beads are incubated with the protein antigen and subjected to extensive proteolysis. The beads are subsequently washed several times to remove the non-epitope fragments. The remaining fragments bound to the antibody contain the epitope. After acid elution from the antibody beads, the epitope containing peptides are identified by MALDI-TOF mass spectrometry (Van de Water *et al.*, 1997).

The major limitations of epitope excision and limited proteolysis epitope mapping assays are that these involve proteolytic cleavage with trypsin. This limits the mapping of the epitope to regions that contain cleavage sites and results in the identification of long peptide sequences (30–60 residues) that poorly define the epitopes. Similarly, the effectiveness of chemical modification is dependent upon label-reactive residues being located within the epitope and on the spacing between these reactive residues (Baerga-Ortiz, *et al.*, 2002).

4.1.14.4 Antigenicity Index

A computer algorithm to predict the topological features of a protein directly from its primary amino acid sequence was described by Jameson (1988). The program generates values for surface accessibility parameters and combines these values with those obtained for regional backbone flexibility and predicted secondary structure. The output of this algorithm, the antigenic index, is then used to create a linear surface contour profile of the protein. As most antigenic sites are located within surface exposed regions of a protein, the program offers a relatively reliable means of predicting potential epitopes (Jameson *et al.*, 1988).

4.1.14.5 SYFPEITHI database

Another method of mapping an epitope is to compare the protein sequence with known epitopes. SYFPEITHI is a database of experimentally mapped T-cell epitopes (Rammensee *et al.*, 1999). This allows to predict whether a candidate epitope is present in a known protein sequence. The SYFPEITHI database takes into consideration the amino acids in the anchor and auxiliary anchor positions of MHC binding peptides as well as other frequent amino acids. Epitope prediction is more powerful for MHC class I alleles for which a large amount of data are available. A reliability of at least 80% in predicting the most likely epitope can be expected. Therefore, the naturally present epitope should be among the top-scoring 2 % of all peptides predicted in 80 % of all cases. For example, if a protein sequence is as long as 400 amino acids, it can be split into 392 possible nonameric peptides. Therefore, the correct epitope should then be among the 8 top-scoring peptides (<http://syfpeithi.bmi-heidelberg.com/scripts/MHCServer.dl/Info.htm#scores>). Hansson *et al.* (2003) used this database to identify T-cell epitopes of the variable region of the Ig heavy chain.

4.1.14.6 Other T Cell Epitope Algorithms

Algorithms are available for the prediction of T cell epitopes. However, a limitation common to these programs is that direct prediction of T-cell epitopes without considering the MHC restrictions could lead to the identification of peptides that are effective in only a narrow range of the population. Furthermore, a peptide may have characteristics of a T cell epitope but may not bind MHC and therefore, not be functional. In addition, functional proteasome cleavage sites are required at the appropriate distance for the peptide to be produced.

Other programs include: EpiPlot 1.0: a program that predicts B and T cell epitopes in proteins from their primary structures. It calculates and plots flexibility, hydrophilicity and antigenicity profiles using 13 different scales (<http://genamics.com/software/files/epiplot1.exe>). EPIPREDICT is a program where the server predicts the MHC class II restricted T cell epitopes based on quantitative matrices (<http://www.epipredict.de/index.html>). RANKPEP is a program that ranks all possible peptides from an input protein sequence by their similarity to a set of peptides known to bind

to a given MHC molecule. Similarity is scored using a Position Specific Scoring Matrix (PSSM) built from a collection of aligned peptides known to bind to that MHC molecule (<http://mif.dfci.harvard.edu/Tools/rankpep.html>).

4.1.15 Hypothesis

Active specific immunotherapy is an attractive approach to cancer therapy in the adjuvant setting. This therapy is intended to boost the host anti-tumour immune response in contrast to passive immunotherapy (Pervin *et al.*, 1997). Active immunisation with anti-idiotypes makes use of internal-image three-dimensional mimicry to simulate tumour antigens. The anti-idiotypic mimics a tumour antigen and is capable of inciting both a humoral and a cell mediated immune response that cross reacts with the tumour (Shoenfeld *et al.*, 1994).

ScFvs are commonly believed to be non-immunogenic. However, previous work in our laboratory had shown that scFvs cross-react strongly with rabbit anti human kappa light chain polyclonal antibodies. This implied the existence of epitopes on human V κ domains that are recognised by rabbit antibodies. Furthermore, sequence comparison showed that whilst the antigen binding canonical loops of human and mouse V κ domains are similar, the corresponding loops of rabbit antibodies have completely different lengths in the L1 and L3 regions (shorter and longer, respectively). On the other hand, the framework region lengths are conserved between human, mouse and rabbit V κ domains. The 3-dimensional structure of the rabbit V domain is unknown. However, the sequence analysis suggested an overall shape similar to humans and mice except for an expansion of the L3 region together with shrinkage of the L1 region. This result raised the possibility of an anti-idiotypic component in the rabbit anti-human V κ reactivity. Furthermore, Lowenadler *et al.*, (1986) suggested that fusion of protein A to human insulin-like growth factor I (IGF-I) can make poor immunogens more immunogenic. The potential use of RAFT3ZZ to stimulate an anti-idiotypic response was therefore explored. An anti-idiotypic response to R3ZZ would probably have no direct therapeutic effect, therefore we attempted to make anti-idiotypic hybridomas and then clone them as scFvs into pEZZ18. These Ab2 scFv ZZ fusions could have triggered a therapeutic Ab3 response.

4.1.16 Aims

To test the potential of R3ZZ to elicit an anti-idiotypic response.

To investigate the immunogenicity of R3ZZ in comparison with the unmodified RAFT3 scFv.

4.2 METHODS

4.2.1 Immunisation

Female CBA mice were used for all immunisations. Mice were obtained from breeding stock at the animal house (Gray Cancer Institute, Mount Vernon Hospital). Standard aseptic techniques were used in all procedures according to the animal house protocols. The mice were housed in MB1 plastic cages (North Kent Plastics) holding a maximum of 20 mice and fed on a diet of standard expanded pellets (B & K Universal). Mice were immunised when they reached between 6 to 10 weeks of age.

A clean operative field was established. Mice were anaesthetised with Enflurane. Once suitably anaesthetised the protein suspension was drawn up into a 1 ml syringe (NHS supplies) and a 23 G x 1" (Terumo™ "blue-hub") needle was fitted to the end of the syringe. Mice were immunised intraperitoneally with a suspension consisting of 25µg protein (scFv, LHM2 mouse mAb or human IgG) in PBS and 100µl Freund's Complete Adjuvant (FCA, Sigma). FCA is a mixture of a non-metabolisable mineral oil, a surfactant (Arlacel A), and mycobacterium (*M. tuberculosis* or *M. butyricum*) cell wall components that potentiate the humoral antibody response to injected immunogens. Adjuvant activity results from sustained release of antigen from the oily deposit and stimulation of a local immune response. Antibody production is enhanced using FCA because of the depot effect and non-specific immuno-potentiation of macrophages by surfactant and mycobacterium. Complete Freund's adjuvant has been found to cause inflammation, necrosis and discomfort and therefore it is limited to the primary immunisation only (Institutional Animal Care & Use Committee Policies and Guidelines, <http://info.med.yale.edu/iacuc/policies/rabbits.html>).

Mice were anaesthetised and repeated boosters administered i.p. every 3 weeks consisting of 25µg protein in PBS and 100µl Incomplete Freund's Adjuvant (IFA) for up to five boosters. IFA has the same oil/surfactant mixture as FCA but does not contain any mycobacterium. It is frequently used as a booster injection as its properties favour humoral immune responses rather than cell mediated responses. Thus, less severe side effects are observed when compared with FCA.

Ten days post immunisation blood samples were taken via the tail vein from immunised mice. Mice were anaesthetised and tails gently heated using an infrared lamp induced tail vein vasodilatation. One hundred μ l blood samples were taken and added to a micro-centrifuge containing 25 μ l 0.5M EDTA, pH 6.8. The samples were stored at -80°C until further analysis. The animals were then returned to their cages after recovery from anaesthesia and observed on a regular basis. Following the completion of the experiment, mice were culled, spleen, liver and kidney harvested and fixed in formal saline for 24 hours. They were processed and stained by H&E to determine the side effects of repeated immunisations (Chapter 5).

4.2.2 ELISA for the Detection of Antibodies Raised Against Proteins

The side effects of R3ZZ were determined by comparison with R3. Tail bleeds from mice immunised with both antibodies were tested by ELISA. Direct ELISA detected antibodies raised against the immunogen.

A ninety six well microtitre plate (Geiner) was coated with 250ng R3 per well in PBS overnight at 4°C . The following morning the plate was washed three times with 200 μ l wash buffer (PBS+ 0.05% Tween 20). Non-specific binding of the sera was prevented by blocking the microtitre plate for 1 hour with 1% bovine serum albumin (BSA) in PBS at 37°C . The plate was then washed, 5 μ l mouse serum titred in Western blot incubation buffer (0.1% BSA plus 0.05% Tween 20 in PBS) and the plate incubated for 2 hours. The plate was washed and 50 μ l of a 1:500 dilution of goat anti-mouse immunoglobulins horseradish peroxidase (HRP) conjugate (Dako) added per well. The plate was incubated for one hour and then washed and stained with 100 μ l per well OPD (Sigma). The reaction was terminated by adding 50 μ l 2M sulphuric acid per well. A plate reader (Biorad) set at 490nm was used to measure the optical density. The antibody titre was determined as the highest serum dilution that gave an OD (490nm) of two times background.

4.2.3 Melanoma Binding

The human melanoma cell line A375M (Giard *et al.*1973) was obtained from the European Catalogue of Animal Cell Culture (ECACC). A375M is an amelanotic melanoma cell line derived from a 54 years old patient with metastatic melanoma.

A375M cells were seeded in 96 well flat bottom microtitre plates at 7×10^3 cells per well and grown to confluence (approximately three days in RPMI plus 10% foetal calf serum). The medium was discarded and the plates dried overnight at 37 °C. The plates were incubated with 250ng R3 per well in culture medium plus azide. Mouse sera were titred and the plates incubated for 2 hours. The plates were washed and 50µl of a 1:500 dilution of goat anti-mouse immunoglobulins horseradish peroxidase conjugate (Dako) was added per well. The plates were incubated for one hour, washed and then stained with OPD. Once stained, 50 µl per well 2M sulphuric acid was added to terminate the reaction. A plate reader (Biorad) set at 490nm was used to measure the optical density.

4.2.4 Specificity for scFv Binding

A series of ELISA assays were carried out to investigate whether the immunised mice that had developed antibodies against R3 had also developed antibodies against other scFvs in the series of scFvs. This was tested by coating 96 well microtitre plates (Geiner) with different scFvs. The scFvs used were R3 (a mouse/human scFv), R2 (mouse scFv), B4 (human scFv) and 4A4 (a mouse/human scFv consisting of monomers, dimers and trimers).

A ninety six well microtitre plate was coated with 250ng scFv per well (R3, B4, R2 or 4A4) in PBS at 4°C overnight. The following morning the plate was washed three times with 200µl wash buffer (PBS-Tween section 4.2.2). Non-specific binding of the sera was prevented by blocking the microtitre plate for 1 hour with 1% BSA in PBS at 37°C. The plate was then washed, 5µl mouse serum titred in Western blot incubation buffer and the plate incubated for 2 hours. The plate was washed and 50 µl of a 1:500 dilution of goat anti-mouse immunoglobulins HRP conjugate was added per well. The plate was then washed and stained as before.

4.2.5 Antigen Binding Inhibition

Surface biotinylation of melanoma cells was carried out using the method of Pelchen-Matthews *et al.* (1991). Briefly, a confluent T75 flask with A375M cells (2.8×10^7) was harvested. The cells were washed twice in PBS. The cell pellet was re-

suspended in 0.5mg/ml L-C biotin (Sigma) in PBS to 1×10^7 cells/ml and placed in a rotary mixer at 4°C for 30 mins. The cells were pelleted for 5min at 1000rpm. The cell pellet was washed twice in 200mM glycine in PBS pH7.5 and resuspended in lysis buffer (2% NP-40 in 20mM Tris-HCl, pH8.0, 150mM NaCl, 2mM EDTA) containing protease inhibitors (Calbiochem) to give 1×10^7 cells/ml. The cells were incubated in a rotary mixer at 4°C for 4 min and then centrifuged at 13000rpm for 5min. The supernatant was retained as biotinylated membrane bound protein extract and stored in aliquots at -80°C.

Two hundred and fifty ng per well R3 was coated in PBS in a 96 well microtitre plate overnight at 4°C. The following morning the plate was washed three times with 200µl wash buffer (PBS-Tween). The plate was blocked for 1 hour with 1% bovine serum albumin at 37°C and then washed with 200µl PBS-Tween per well. The plate was incubated with 50µl biotinylated extract per well, 5µl serum was titred and the plate incubated at room temperature for 2 hours. The plate was washed with wash buffer. Fifty µl of a 1:1000 dilution of streptavidin horseradish peroxidase conjugate (Dako) was added per well. The plate was incubated for one hour and then washed three times with PBS. The plate was stained with 100 µl per well OPD and kept in the dark until colour developed. Once stained 50µl per well 2M sulphuric acid was added to terminate the reaction. A plate reader (Biorad) set at 490nm was used to measure the optical density.

4.2.6 IgG Titre

Five µl mouse blood and 5µg mouse IgG standard (Sigma) was titred in PBS in a 96 well microtitre plate and incubated overnight at 4°C. The following morning the plate was washed three times with 200µl wash buffer (PBS-Tween). The plate was blocked for 1 hour with 1% BSA in PBS at 37°C and then washed with 200µl PBS-Tween per well. Fifty µl of a 1:1000 dilution of goat anti mouse immunoglobulins HRP (Dako) was added per well. The plate was incubated for one hour and then washed three times with PBS-Tween. The plate was stained with 100 µl per well OPD. The plate was kept in the dark until colour developed. Once stained 50µl per well 2M sulphuric acid was added to terminate the reaction. A plate reader (Biorad) set at 490nm was

used to measure the optical density. The antibody titre was determined as the highest serum dilution that gave an OD of two times background.

4.2.7 Antigen Specificity – Analysis on Panel of scFvs by Western blot

Glycerol stocks were used to inoculate bacterial overnight cultures in 1.5ml LB with 100µg/ml ampicillin (LB Amp₁₀₀) and 2% D-glucose. The bacteria were grown at 30°C overnight. The following morning the bacteria were diluted 1:100 in fresh LB Amp₁₀₀ plus 2% D-glucose and incubated for 7 hours at 30°C. The bacteria were centrifuged for 10 min at 3000rpm and the supernatant aspirated off. The bacteria were resuspended in 1.5ml LB Amp₁₀₀ and 500µM IPTG and grown overnight at 30°C. The following morning the bacteria were centrifuged at 3000 rpm and the supernatant retained for Western blot analysis.

Fifteen percent SDS-PAGE gels were poured following manufacturers instructions [Chapter 2 Section 2.19]. Samples were prepared, electrophoresed and proteins transferred onto nitrocellulose membranes and blocked overnight in *blocking buffer* [Chapter 2 Section 2.20]. The following morning the nitrocellulose was washed three times with *wash buffer* [Chapter 2 Section 2.20] and incubated for two hours with a 1:1000 dilution of mouse serum from mice immunised with either R2, R3, B4 or R3ZZ. Following the incubation filters were washed in *wash buffer* and then incubated with goat anti-mouse IgG (Fc-specific) alkaline phosphatase conjugate (Sigma) 1:1000 for two hours.

In order to control for differences in scFv expression yields the following stains were used as appropriate:

- Mouse anti-human *c-myc* 9E10 supernatant (Sigma) (goat anti-mouse IgG AP).
- Rat anti-yeast α -tubulin mAb Yol 1/34 (Serotec) (goat anti-rat IgG AP)
- Rabbit anti-human kappa light chain polyclonal antibody (Dako) (goat anti-rabbit IgG AP)

Filters were then washed and developed in development buffer plus reagents A and B (Biorad). Following sufficient development the reaction was terminated by washing the filters under tap water.

4.2.8 Construction of R2 without c-Myc

R2 without *c-myc* was cloned to determine whether the epitope was located on the artificial *c-myc* detection tail. The R2 scFv (Kupsch *et al.* 1995) was modified by amplifying the R2 sequence consisting of a gene 3 secretion leader, mouse V_H and V_K and a polyglycine serine linker using primers R586 and R38. The R2 scFv was cloned *Hind*III / *Eco*RI into vector pUC19 (Yanish-Perron *et al.*, 1985). The clones with the correct size insert were sequenced to confirm the correct structure and stored at –80°C [Chapter 2 Section 2.2.1].

4.2.9 Construction of R3 without c-Myc

RAFT3 without *c-myc* was cloned to determine whether an epitope was located on the human V_K domain. It was cloned *Hind*III *Eco*RI into pUC19 (Yanisch-Perron *et al.*, 1985). The R3 without *c-myc* consisted of the gene 3 secretion leader, mouse V_H polyglycine serine linker and human V_K. Primers R38 and R666 were used for PCR. Once cloned the clones were screened in a colony lift [Chapter 2 Section 2.1.13] with rabbit anti-human kappa 1:1000 (Dako) followed by goat anti-rabbit IgG alkaline phosphatase 1:1000 (Sigma). Positive clones were sequenced to confirm the correct structure and stored as glycerols at –80°C [Chapter 2 Section 2.2.1].

4.3 RESULTS

4.3.1 Immunisation of CBA Mice

At first mice were immunised with R3ZZ and R3 as a negative control. Following the initial immunisation with complete Freund's adjuvant, 5 boosters with incomplete Freund's adjuvant were administered at three-week intervals.

Following 3 immunisations with both R3 and R3ZZ or adjuvant only mice generally developed distended abdomens. This was quite severe in some mice and virtually non-existent in other mice in the same group. This is a known side effect of intraperitoneal injection of proteins with Freund's adjuvant (Tung *et al.*, 1976).

After the completion of immunisation, the mice were subject to a post mortem. Post mortem of the mice revealed a large quantity (up to 2ml) of ascites fluid in the abdominal cavity as expected (Tung *et al.*, 1976). No T cells were seen in the ascetic fluid. The spleen, liver and kidney were excised and examined in histology as described in Chapter 5.

4.3.2 Detection of Antibodies Raised Against Proteins

Analysis of sera from immunised mice by ELISA detected antibodies raised against the immunogen. Figure 4.7 illustrates the reactivity of antibodies raised against the antigen over the course of immunisations. The 5 immunised mice from each group gave similar results.

The data shown in Figure 4.8 are from a representative mouse where the largest quantity of mouse serum was available for further studies. This was an important consideration as mouse sera were precious and a potential limiting factor for the study. However, for each experiment sera from other mice were also tested.

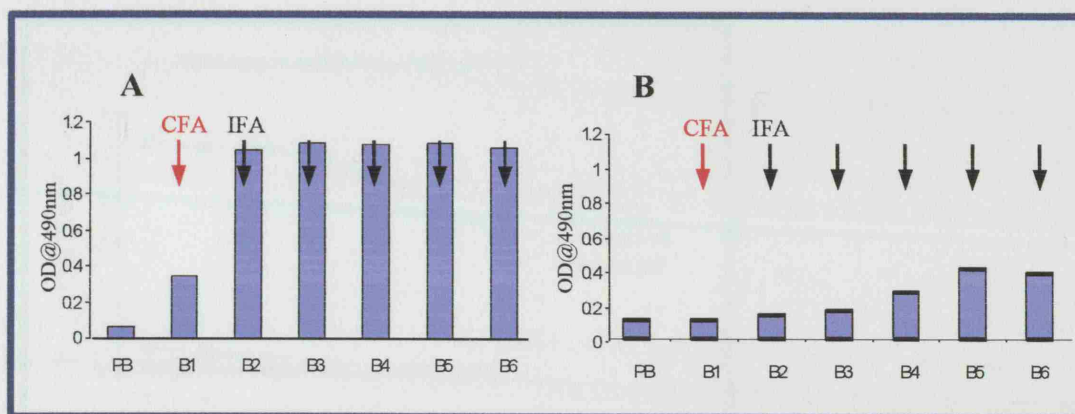


Figure 4.7 Reactivity of sera from mice immunised with R3 (A) and R3ZZ (B) with R3 in ELISA. PB: Pre immune serum, B1-B6: sera obtained after initial immunisation (B1) and boosters 1-5 (B2 to B6). CFA, Complete Freund's Adjuvant; IFA, Incomplete Freund's Adjuvant.

The ELISA showed that antibodies were raised against both R3 and R3ZZ. However, binding to R3 protein was considerably higher by sera from mice immunised with R3 in comparison with mice immunised with R3ZZ (Figures 4.7 and 4.8). This result was contrary to our expectations. Furthermore, the results showed that sera of the R3 immunised mice reach optimal antigen binding following a single booster, whereas the sera of R3ZZ immunised mice reached a much lower binding plateau following the fourth booster. Therefore 3 boosters were deemed sufficient for subsequent immunisations with scFv. The anti-R3 antibody titre was 1:25,000 after the third booster. The serum and IgG titre in R3 and RAFT3ZZ immunised mice was similar (2 and 0.75mg/ml, respectively).

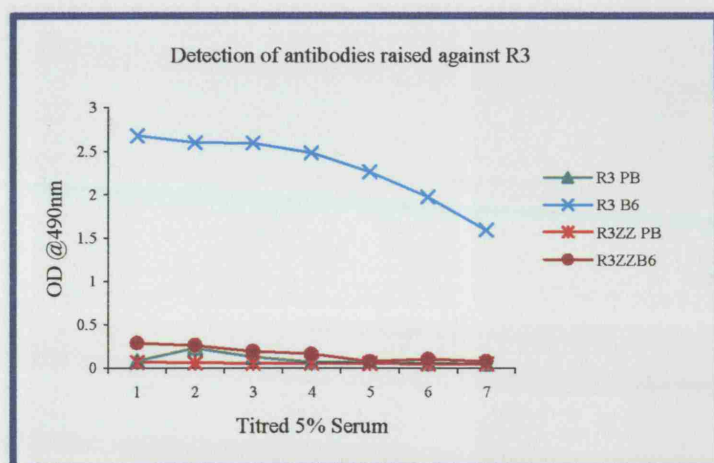


Figure 4.8: Comparison of the effect of immunising mice with R3 and R3ZZ. Sera obtained before immunisation (pre bleed, PB) and from the last immunisation (B6) were tested for reactivity with R3 in direct ELISA. A one in 20 dilution of serum was titred two-fold on an ELISA plate coated with R3 scFv.

4.3.3 Protein Binding by Sera

RAFT3 and RAFT3ZZ have identical mouse V_H linker and human V_K sequences but different detection tails (human c-myc or ZZ, respectively). The ability of sera to bind scFvs was tested in Western blot. The Western blot in Figure 4.9 shows that sera from R3 immunised mice were able to bind several other scFvs (all with c-myc tag) including the mouse scFv RAFT2 and the human scFvs B3 and B4. This is described in more detail in section 4.3.11

4.3.4 Antigen Specificity – Analysis on Panel of Scfvs by Western blot

The results of Western blots confirmed that mouse serum from R3 immunised mice could detect scFvs. However, initially detection of non-specific proteins was a problem. These were visible only on blots stained with immune sera and were presumed to be immunodominant *E.coli* proteins that were present at low levels in the scFv preparations used for immunisations.

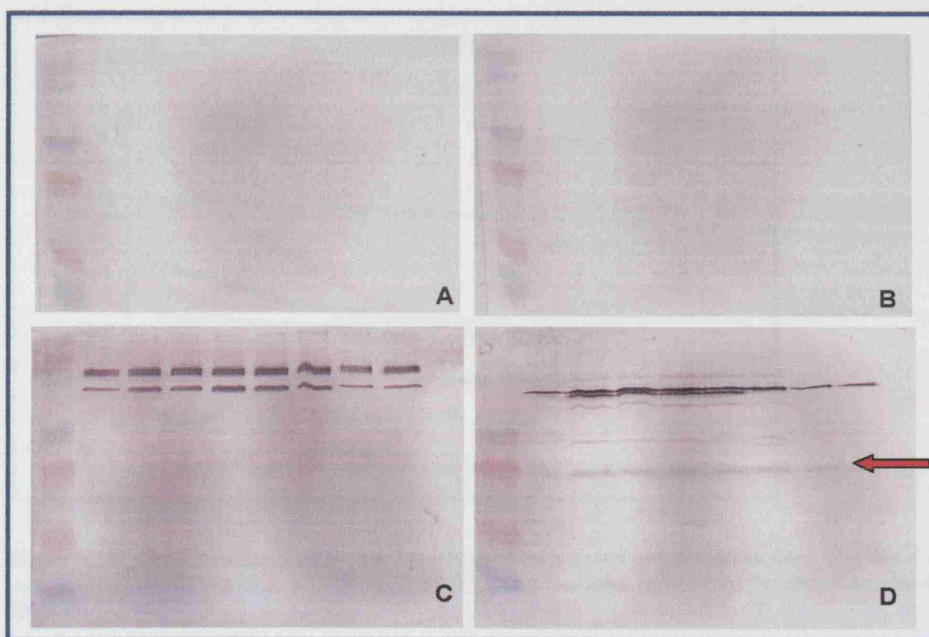


Figure 4.9: Supernatants of a panel of scFvs were analysed by Western blot and detected with mouse serum. : Km, 3A11, R2, R3, B3, B4, 4A4, 5B3 and 4F2 scFv. The mobility of scFv is indicated by an arrow. A: R3ZZ Pre-bleed, B: R3 Pre-bleed, C: R3ZZ Post-bleed, D: R3 Post-bleed.

4.3.5 Melanoma Binding

The ability of R3 scFv to mediate binding of mouse immune sera to the high molecular weight antigen was tested by ELISA on A375M melanoma cells. The results show that following immunisation the sera could be used to detect R3 binding to high molecular weight antigen when compared with pre-immune sera (Figure 4.10). These results confirm the specificity of serum binding to R3 and that R3 is more immunogenic than the R3ZZ fusion molecule.

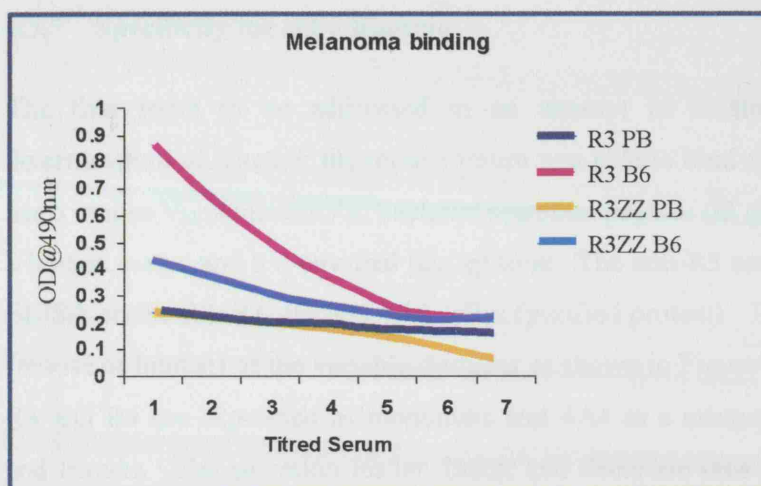


Figure 4.10: ScFv mediated melanoma binding by sera pre-and post-immunisation. Pre-bleed (PB) and post immunisation sera (B6) from both R3 and R3ZZ immunised mice were tested for melanoma binding via R3 by ELISA.

4.3.6 Epitope Mapping

The results obtained from the immunisation of mice with R3, R3ZZ were unexpected and in contrast to the common belief that scFvs are poor immunogens. This triggered a series of experiments aimed at locating the epitope(s) responsible for the immunogenic properties of scFvs. Figure 4.11 illustrates the linear and 3D structure of scFv.

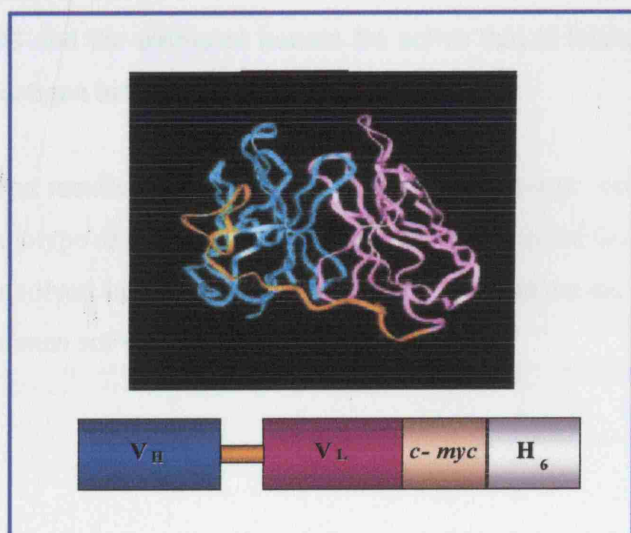


Figure 4.11: Typical 3D and linear structure of the scFv.

4.3.7 Specificity for scFv Binding

The first issue to be addressed in an attempt to locate the epitope was the determination of whether the mouse serum was able to bind scFvs other than R3. R3 has a mouse V_H, a human V_K, bacterial secretion peptide (fd gene III), a (G₄S)₃ linker, a human c-myc and a c-terminal his₆ epitope. The anti-R3 serum was tested in direct ELISA on the R2, R3, B4 and 4A4 scFvs (purified protein). These differ in the origin (mouse or human) of the variable domains as shown in Figure 4.12. Furthermore, R2, R3 and B4 are expressed as monomers and 4A4 as a mixture of monomers, dimers and trimers. The secretion leader, linker and detection tails of R2, B4 and 4A4 are identical with R3.

The results showed that serum obtained from R3 immunised mice bound to R3, B4 and R2, but not to 4A4. Sera from mice immunised with R3ZZ bound weakly to R3 and R2 but not to B4 and 4A4. This result suggests that the epitope could be located at the bottom of the scFv facing away from the antigen binding site. Furthermore, this location would also permit simultaneous binding of R3 scFv to HMW-MAA and mouse anti-scFv antibodies without steric inhibition (Figure 4.10). The R2, R3 and 4A4 scFvs bind the same epitope on HMW-MAA whereas the B4 scFv binds an unrelated melanoma specific antigen (Kupsch *et al.*, 1999). The cross-reactivity of anti-R3 mouse serum is also consistent with the existence of a shared epitope on the R3 and the untreated human B4 scFvs that is located at a distant from the paratope (antigen binding site).

The results appear to exclude the linker, c-myc, conserved human V_K residues and idiotype as locations (see discussion) and pointed towards a role of a region that is not involved in antigen binding by scFv and present on both the mouse scFv R2 and the human scFv B4.

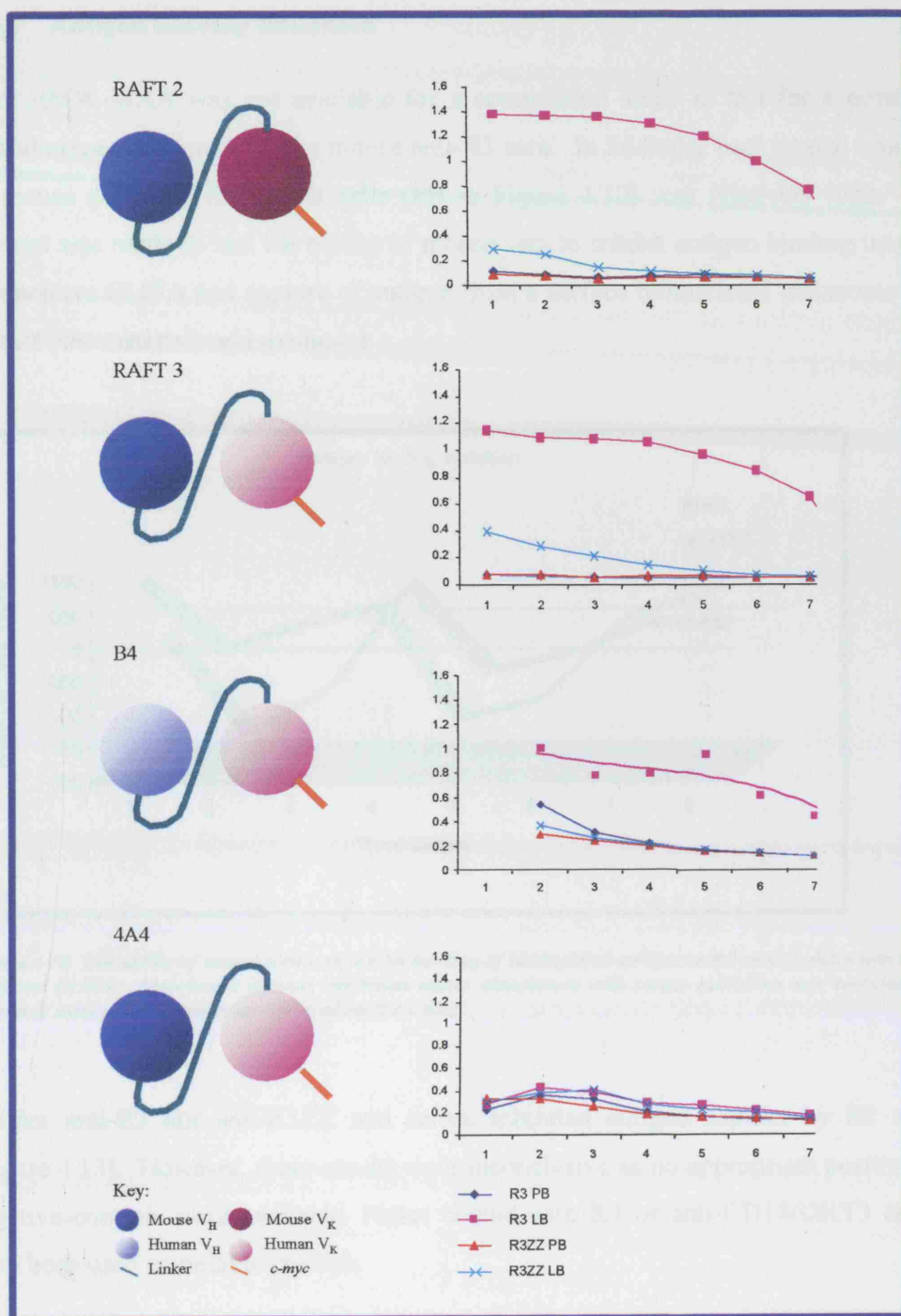


Figure 4.12: Mouse anti R3 and R3ZZ sera binding pure scFvs in direct ELISA. Two fold serial dilutions were made starting with 5% serum (x-axis) and the absorbance (y-axis) measured at 490nm. The purity in SDS-PAGE and melanoma binding (detected by 9E10) of the scFv preparations was similar.

4.3.8 Antigen Binding Inhibition

Pure HMW-MAA was not available for a competition assay to test for a potential anti-idiotypic component in the mouse anti-R3 sera. In addition, background binding of mouse serum to melanoma cells (PB in Figure 4.10) was relatively high. An attempt was made to test the ability of mouse sera to inhibit antigen binding using a competitive ELISA and capture of antigen from a surface biotinylated melanoma cell extract (see materials and methods).

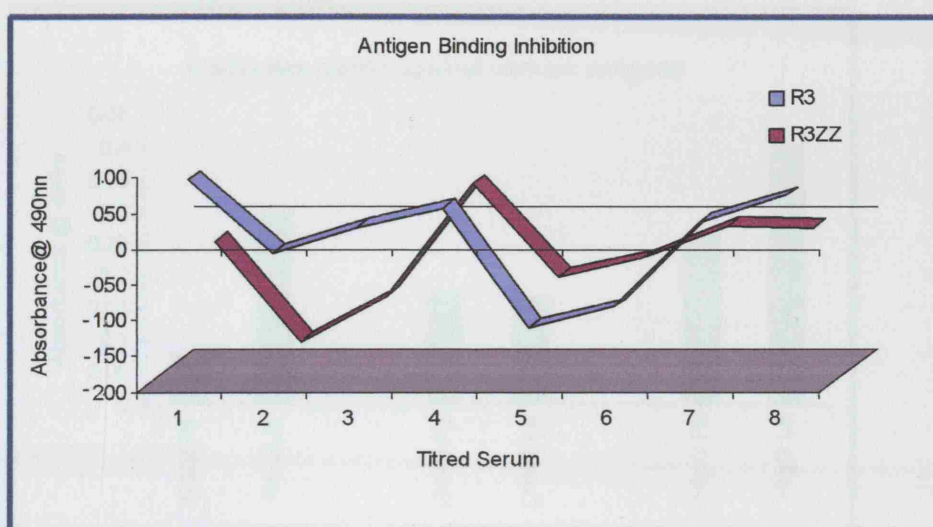


Figure 4.13: The ability of mouse serum to inhibit binding of biotinylated antigen to R3 coated plates was tested in Direct ELISA. Absorbance without inhibition minus absorbance with serum inhibition was calculated at 490nm (y axis) and 5% mouse serum titred on the x axis.

Neither anti-R3 nor anti-R3ZZ anti serum inhibited antigen capture by R3 scFv (Figure 4.13). However, these results were inconclusive as no appropriate positive or negative controls were available. Plates coated with R3 or anti-CD18/OKT3 could have been used as suitable controls.

4.3.9 Immunogenicity of Parent Monoclonal Antibody and Human IgG

The immunogenicity of the whole parent monoclonal antibody of R3 scFv, LHM2 (mouse IgG₁) and human IgG were tested by immunising CBA mice. Pre and post immune sera were analysed in ELISA using R3 coated microtitre plates. Serum raised against LHM2, the whole monoclonal antibody did not cross react with R3

(Figure 4.14). Sera raised against human IgG showed weak cross reactivity, similar to anti-R3ZZ sera. This suggested the existence of a weakly immunogenic epitope shared between R3, R3ZZ and human IgG and presumably located on the human V κ domain of R3 (see discussion). This is the only region that the three immunogens have in common. This also excludes the linker, *c-myc*, *his*₆ sequence and artificial junctions (potential neoepitopes) on the scFv as well as the gene III secretion leader (that could be incompletely cleaved off in some scFv molecules) as the sole epitope.

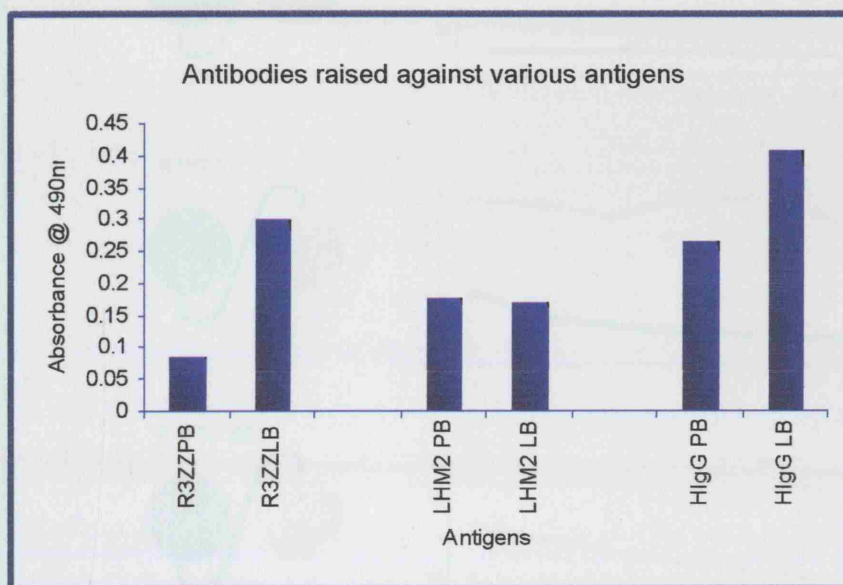


Figure 4.14: Binding of Pre and Post immune sera from mice immunised with R3ZZ, LHM2 and human IgG (HlgG) to pure R3 scFv tested in ELISA.

4.3.10 Immunogenicity of other scFvs

The immunogenic properties of other scFvs were determined by immunising CBA mice with the mouse R2 and human B4 scFvs. The mice were immunised once with Complete Freund's Adjuvant (CFA) and three boosters with Incomplete Freund's Adjuvant (IFA). Only three boosters were administered, as this had been shown as sufficient to induce an immune response with R3 (Figure 4.7A). The sera prior to immunisation (PB) and following 3 boosters (LB) were tested for R3 binding by ELISA as in section 4.3.6. The results are shown in Figure 4.15.

Sera of mice immunised with R3 and R2 (mouse/human and mouse, respectively) cross-reacted more strongly with R3 sera than sera of R3ZZ and B4 (human)

immunised mice. Since cross-reactivity of sera from R2 immunised mice with R3 was observed it was concluded that the epitope could not be the human V κ region alone. This suggested that there must be a second epitope(s) involving linker, the *c-myc* or the his₆ tail.

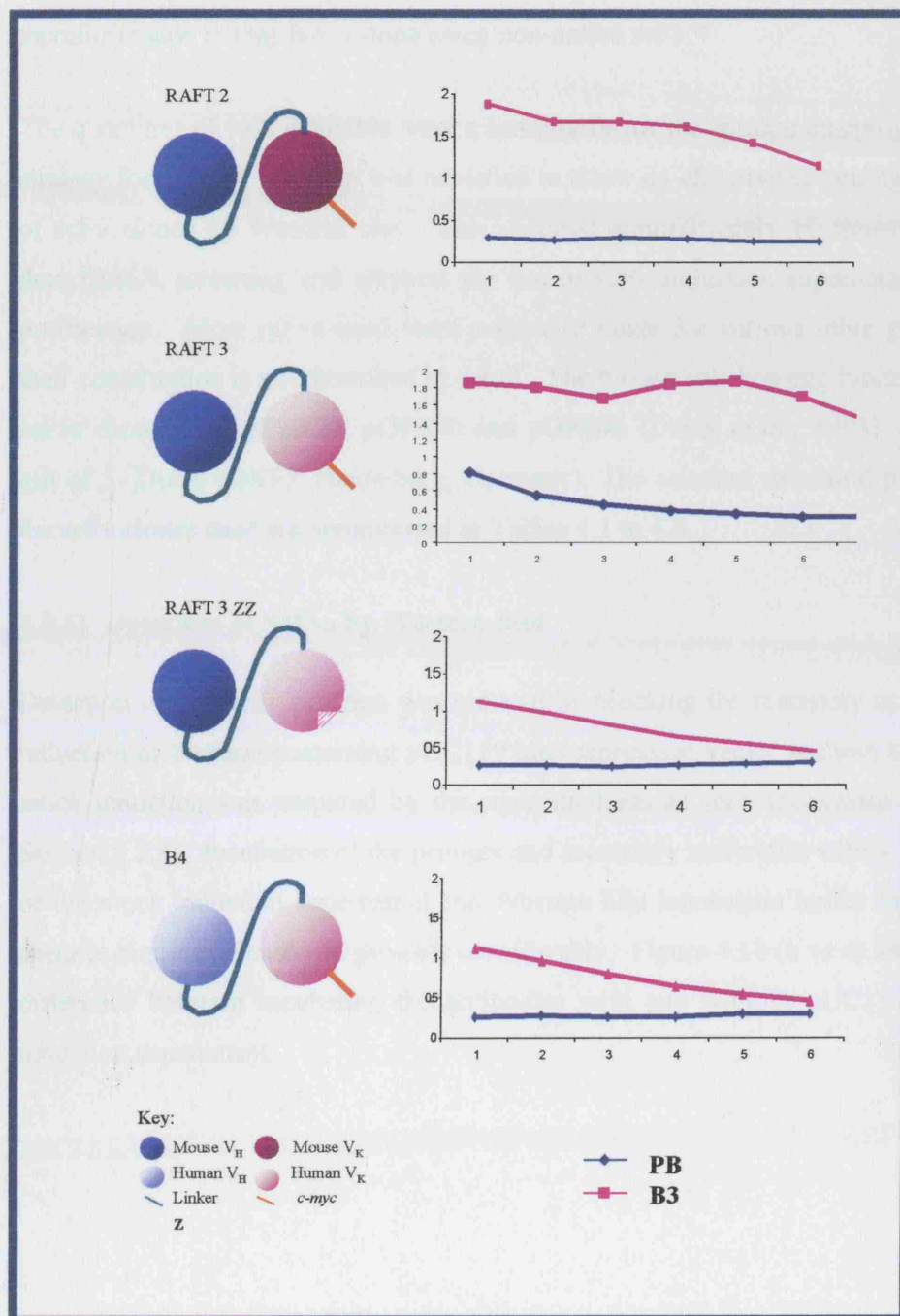


Figure 4.15: The ability of mouse sera to bind pure R3 in ELISA. The binding of pre bleeds (PB) and serum following 3 boosters (B3) is shown for R2, R3, R3ZZ and B4.

Up to this point the results obtained with native scFv (direct ELISA, melanoma binding, competition binding) appeared to exclude all regions tested except the (G₄S)₃ linker or the junction of the V domains as a location for the epitope. An attempt was therefore made to map the epitope using non-native scFv.

The quantities of sera available were a limiting factor for epitope mapping. Thus the strategy for epitope mapping was modified to allow an effective screening on a panel of scFv clones by Western blot. This required approximately 10 times less serum than ELISA screening and allowed the use of scFv induction supernatants without purification. Most scFvs used were originally made for various other projects and their construction is not described in detail. The mouse anti-hen egg lysozyme (HEL) scFvs encoded by pSEX20, pOPE40 and pOPE90 (Dubel *et al.*, 1993) were a kind gift of S. Dubel (DKFZ Heidelberg, Germany). The relevant structural properties of the scFv clones used are summarised in Tables 4.1 to 4.6.

4.3.11 Detection of ScFvs by Western blot

Detection of non-scFv proteins was reduced by blocking the reactivity using a mock induction of bacteria containing pUC119 his (expression vector without insert). The mock induction was prepared by the same methods as scFv inductions [Chapter 2 Section 2.2.3]. Incubation of the primary and secondary antibodies with a 1:1 dilution of the mock induction supernatant and Western blot incubation buffer reduced non-specific binding of bacterial proteins considerably. Figure 4.18 (b vs c) illustrates the difference between incubating the antibodies with and without pUC119 his mock induction supernatant.

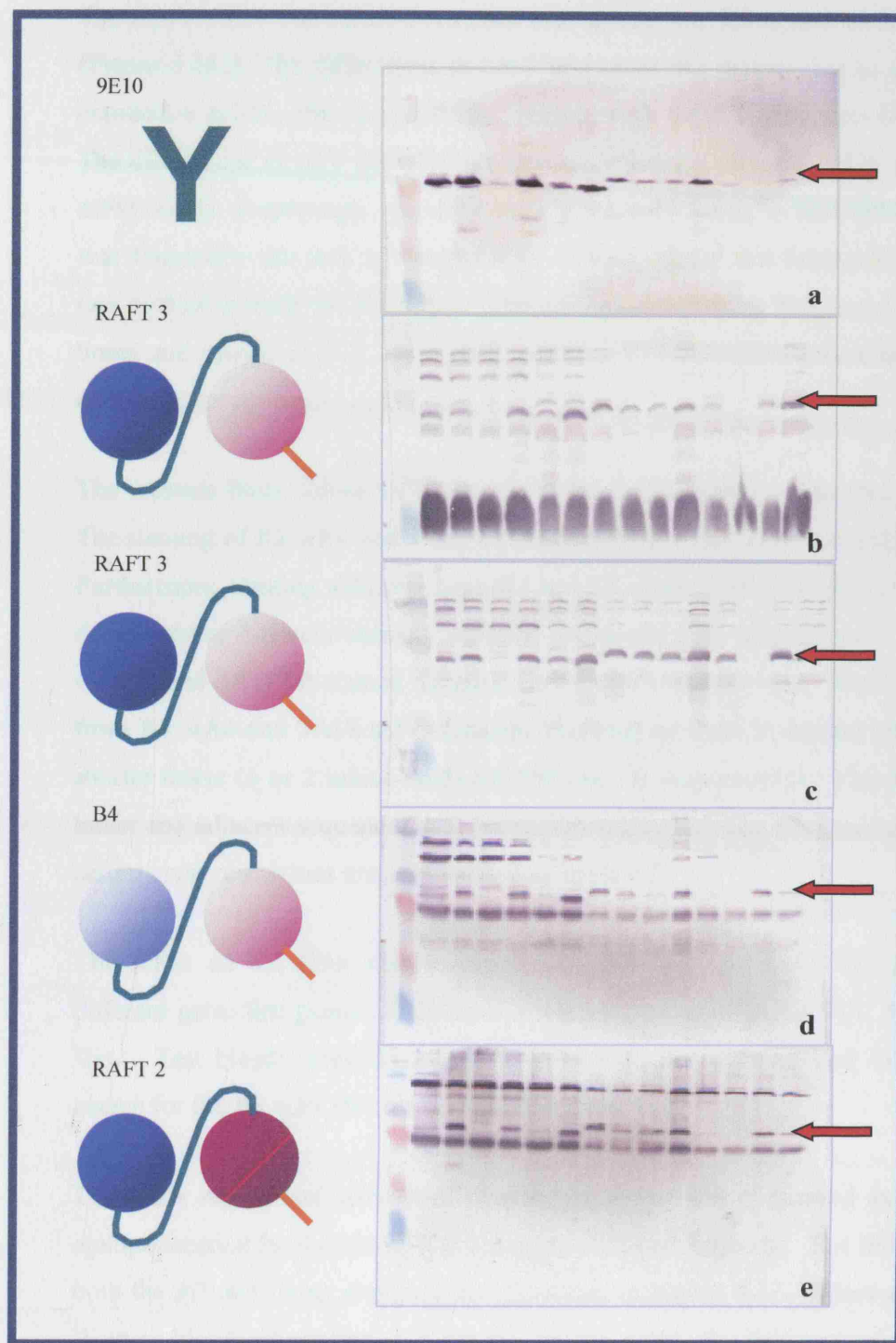


Figure 4.16: Comparison of filters stained with 9E10 (a), anti-R3 mouse serum incubated in Western incubation buffer (b), anti-R3 mouse serum plus pUC119 his mock induction (c) and serum from mouse immunised with B4 (d) and R2 (e) plus pUC119 his mock induction. The scFvs were loaded in the following order: KM, B3, B4, R2, R3, R3DB, R4, 3A11, 3A11DB, 3A11TB, 4A4, 4A4TB, pUC119, 4F2 and 5B3. The filters were stained with 9E10 supernatant (a) or a 1:1000 dilution of mouse sera. Arrows indicate the mobility of scFv.

The results show that serum from mice immunised with R3 is able to detect all scFvs (Figure 4.16c). The differences in band intensities are mainly due to differing scFv expression yields. This is shown by staining with 9E10 supernatant (Figure 4.18a). The differences in scFv mobility are due to differing CDR and linker lengths of the scFvs tested. Surprisingly, the 4A4 scFv previously found to be negative in ELISA was detected by the sera in Western blot. This suggested that there may be more than one epitope present on the scFv. The epitope detected in Western blot should be linear and denatured (e.g. *c-myc* tail), whereas ELISA detects native and non-native epitopes.

The western blots shown in Figure 4.16 are representative of several experiments. The staining of R2 scFv was weak in all experiments due to its low expression yield. Furthermore, staining with sera from B4 and R2 immunised mice (Figure 4.16d and e) demonstrated different staining patterns compared with anti-R3 sera. The R3DB, 4A4TB and 3A11TB stained negative with anti-R2 and B4 sera. These clones differ from R3, 4A4 and 3A11 scFvs (stained positive) by their V domain orientation and shorter linker (5 or 2 amino acids for DB and TB respectively). This suggested the linker and adjacent sequences as a potential neoepitope. The structural properties and serum cross reactivities are summarised in Table 4.2.

The scFvs on the filter also included 4F2 and 5B3 that have V κ s derived from different germ line genes. Different human V κ s tested included V κ 1, V κ 2, V κ 4 and V κ 6. Test bleeds from B4 immunised mice do not stain HuV κ 1 DPK9 regions, except for the R3 scFv (B4 has a DPK26 derived V κ).

The linker region and adjacent V domain sequences was eliminated as the potential epitope location by the use of R4, a domain swapped antibody. The linker region on both the 3D and linear structure are illustrated in Figure 4.17. When denatured for Western blot the linear structure for R3 and R4 varies, the linear structure of R3 and R4 is illustrated in Figure 4.18. The different linkers tested included G₄A(G₄S)₂, G₄S₃, G₄S and α tubulin linkers.

The mouse anti-hen egg lysozyme (HEL) scFv encoded by vector pSEX20 (Table 4.1) is of particular interest. This scFv reacted with all mouse sera including the anti-

human B4 scFv serum in spite of its murine V domains and entirely different artificial sequences (*pel* B secretion leader, α -tubulin linker, gene III fusion instead of *c-myc*/*his*₆ detection tag).

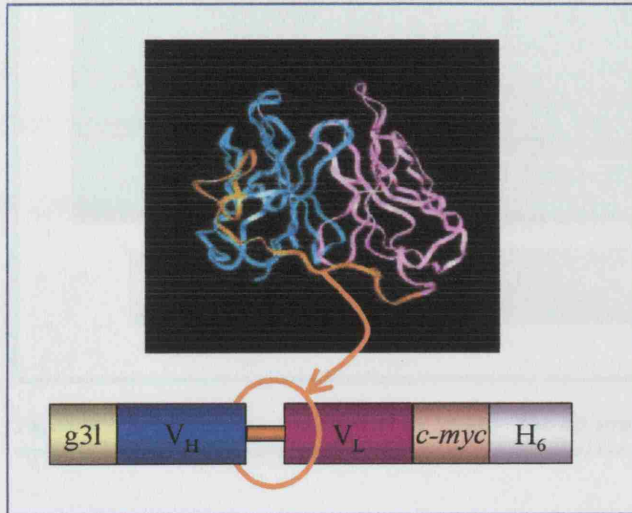


Figure 4.17: 3D and linear structure of scFv with the linker region highlighted. The majority of the scFvs tested has a poly glycine serine linker (Gly4Ser)₃. Clone anti-HEL expressed in vector pSEX20 allowed the polyglycine linker to be excluded as the epitope as it has an α -tubulin linker. The R4 scFv allowed an epitope spanning the linker V domain junctions to be excluded as the epitope would be disrupted (Figure 4.20)

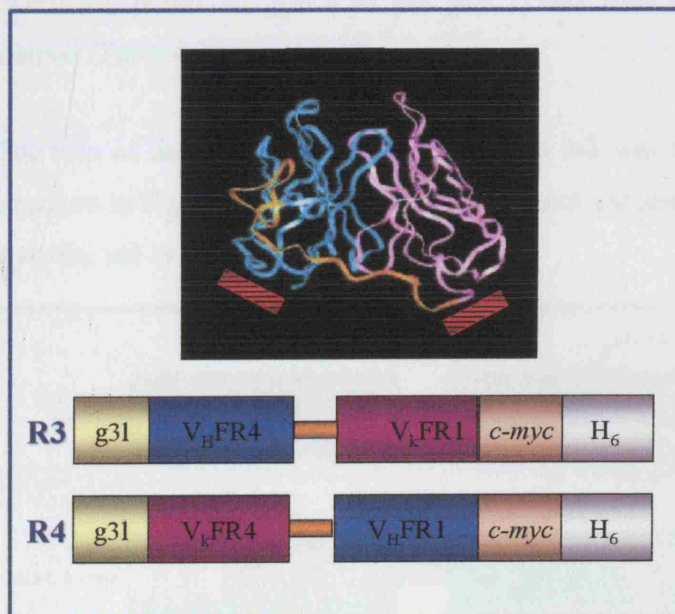


Figure 4.18: 3D and linear structure of scFv with the bottom of the scFv highlighted. The region at the bottom of the scFv were excluded as potential epitope using R4. R4 is a domain swap antibody and its structure compared to R3 is shown in the linear structure. The bottom of the scFv region is disrupted in R4 thereby allowing both these regions to be excluded as the potential epitope site.

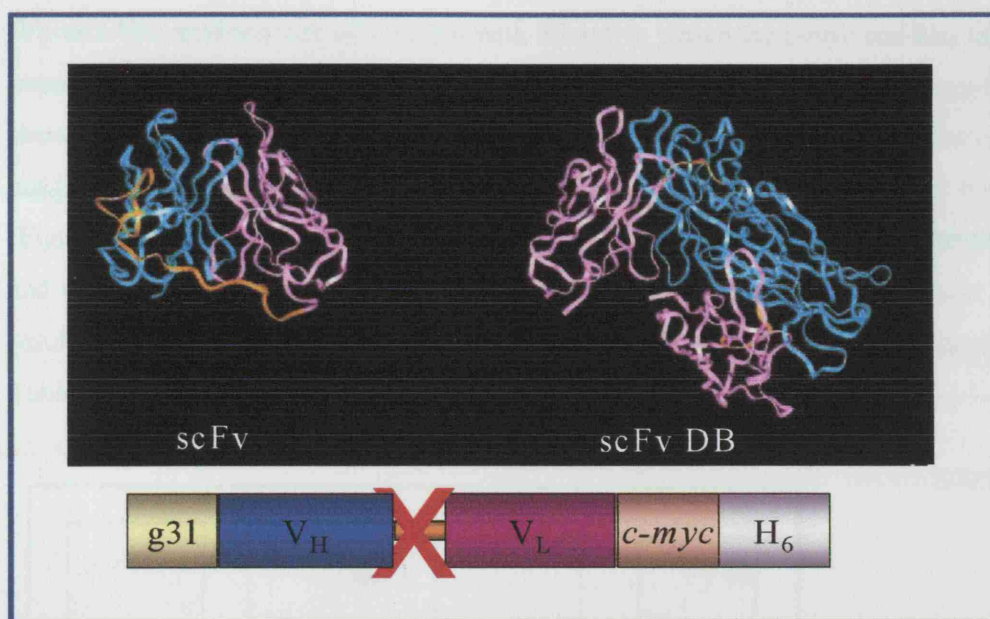


Figure 4.19: 3D structure of R3 scFv and R3DB. The 2D structure shows that the linker is shortened to 5 amino acids and thus the linker was excluded as the potential site of the epitope.

Figure 4.19 illustrates the 3D structure of the R3 scFv and R3DB. Several other scFvs with shortened linkers tested (e.g. 3A11 DB, 3A11 TB) were also stained by immune sera (Table 4.2). In addition, 4 mouse anti-HEL scFvs encoded by vectors pOPE 40, pOPE 90, pSEX20 and pUC119his with the alpha tubulin linker were stained (Table 4.3).

The role of the human *c-myc*/his6 detection tail was investigated next. The linear structure in Figure 4.20 illustrates the structural variations between scFvs with the *c-myc*/his6 tail or kappa constant domain.

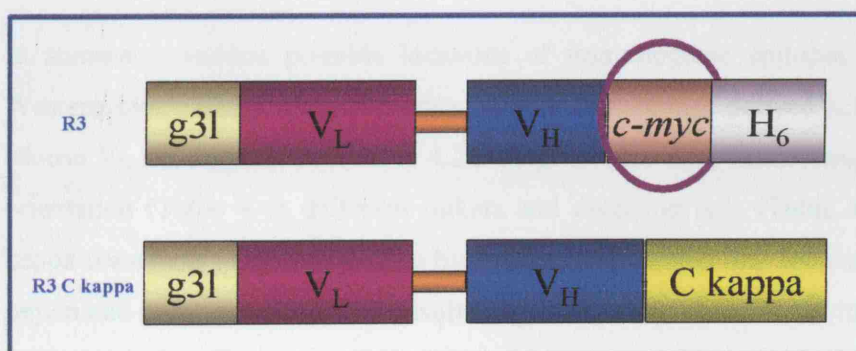


Figure 4.20: Linear structure of R3 and R3 C kappa.

Western blot analysis was carried out with R3 scFvs where the *c-myc* and his₆ tail had been replaced with either mouse or human kappa constant domains. The results are shown in Figure 4.21. 9E10 staining was unable to detect any scFvs as the *c-myc* was not present. Serum from mice immunised with R3 detected R3moCκ but not R3huCκ (Figure 4.21). Serum from mice immunised with B4 showed opposite staining pattern and mice immunised with R2 did not detect either mouse or human Cκ clones. The results of the various epitope mapping assays by Western blot are summarised in Tables 4.1 to 4.6.

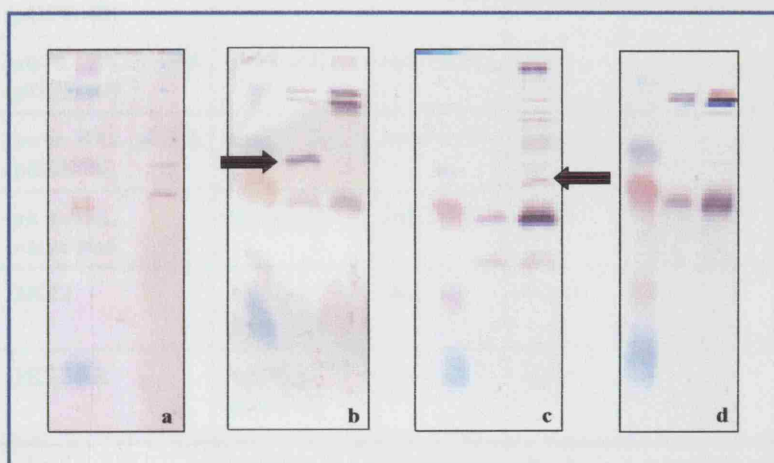


Figure 4.21: Serum binding to R3 moCκ (lane 2) and R3 huCκ (lane 3). Filters were stained with 9E10 (a) or anti-R3 (b), anti-B4 (c) and anti-R2 (d) mouse immune sera. The specifically stained R3Cκ bands are indicated by arrows.

4.3.12 Summary

In summary, various possible locations of immunogenic epitopes were tested by Western blot. These are summarised as follows: Mouse derived scFvs (Table 4.1), Mouse V_H and human V_κ (Table 4.2), *c-myc* (Table 4.3), linker lengths and domain orientation (Table 4.4), different linkers and detection tails (Table 4.5) and scFv C kappa constructs (Table 4.6). The human V_κ was studied first followed by the linker region and *c-myc*/his₆ tail. The results suggest a complex anti-scFv immune response that cannot be directed against the G₄S₃ linker or the *c-myc*/his₆ tail only. For example, serum from mice immunised with the human scFv B4 cross reacts with anti-

HEL scFv encoded by pSEX20, i.e. a mouse scFv with a different linker, secretion signal and with no *c-myc/his₆* tail (Table 4.1).

ScFv	V _H	V _κ	Linker	Mouse Test Bleed		
				R2	R3	B4
R2	mo V _H IIA Vgam3.8	moVκ1- 117*01	(G ₄ S) ₃	✓	✓	-
R2 minus c- myc	mo V _H IIA Vgam3.8	moVκ1- 117*01	G ₄ A (G ₄ S) ₂	✓	✓	✓
mo α HEL c-myc (pOPE 40)	mo V _H IB	mo Vκ V	α-tub	-	-	✓-
mo α HEL his6 (pOPE 90)	mo V _H IB	mo Vκ V	α-tub	✓	✓	✓
mo α HEL gene3 (pSEX 20)	mo V _H IB	mo Vκ V	α-tub	✓	✓	✓
mo α HEL c-myc/his6	mo V _H IB	mo Vκ V	α-tub	-	✓	✓
OKT3	mo V _H 2 gene H30	mo Vκ4	(G ₄ S) ₃	✓	✓	✓
OKT3DB	mo V _H 2 gene H30	mo Vκ4	G ₄ S	✓	-	✓
OKT3 hu Cκ	mo V _H 2 gene H30	mo Vκ4	(G ₄ S) ₃	ND	-	-

Table4.1: Summary of the results of Western blots of mouse derived scFvs.

4.3.13 Mouse V_H and human Vκ ScFv

Table 4.2 summarises the results of detection of scFvs with a mouse V_H and human Vκ domains with mouse immune sera. The R3 scFv reacted with all test bleeds whereas the R3DB and R4 did not stain with the B4 test bleed. No difference in staining was observed with the 3A11, 3A11DB and 3A11TB series. No differences in the staining characteristic were observed with the different human Vκ subgroups tested. The different linkers do not demonstrate any effect on the staining patterns with the different mouse sera.

ScFv	V _H	V _K	Linker	Domain Orientation	Rabbit α Human κ chain Polyclonal	Mouse Sera		
						R2	R3	B4
R3	mo V _H 2A Vgam3.8	Hu V _K 1 DPK9	G ₄ A (G ₄ S) ₂	HL	+	+	+	+
R3DB	mo V _H 2A Vgam3.8	Hu V _K 1 DPK9	G ₃ AS	LH	+	+	+	-
R4	mo V _H 2A Vgam3.8	Hu V _K 1 DPK9	(G ₄ S) ₃	LH	+	+	+	-
3A11	mo V _H 2A Vgam3.8	HuV _K 6 DPK26	G ₄ A (G ₄ S) ₂	HL	+	+	+	+
3A11 DB	mo V _H 2A Vgam3.8	HuV _K 6 DPK26	ASG ₃	LH	+	+	+	+
3A11TB	mo V _H 2A Vgam3.8	HuV _K 6 DPK26	AS	LH	+	+	+	+
4A4	mo V _H 2A Vgam3.8	HuV _K 4 DPK24	G ₄ A (G ₄ S) ₂	HL	+	+	+	+
4A4TB	mo V _H 2A Vgam3.8	HuV _K 4 DPK24	AS	LH	+	+	+	+
4F2	mo V _H 2A Vgam3.8	HuV _K 4 DPK24	G ₄ A (G ₄ S) ₂	HL	+	+	+	+
5B3	mo V _H 2A Vgam3.8	Hu V _K 2 DPK15	G ₄ A (G ₄ S) ₂	HL	+	+	+	+

Table 4.2: Summary of the results of Western blots of scFvs with a mouse V_H and a human V_K. No differences in staining patterns can be attributed to the different linkers or V_K domains. Rabbit anti-human kappa chain polyclonal antibody (DAKO) and 9E10 was used to control for differences in scFv expression levels (data not shown).

4.3.14 Detection of Epitope on *c-myc*

A range of scFvs with and without *c-myc*/his₆ detection tail was compared to determine the role of this commonly used sequence in triggering an immune response. To this end, scFvs were used in which the human *c-myc* / artificial his₆ sequence had been replaced by other detection tags such as the kappa constant domain or fd gene III. Appropriate detection antibodies (rabbit anti-human kappa chain, rat anti-yeast α -tubulin Mab Yol3A) were used to control for differences in expression yield (Chapter 4 Section 4.2.7).

Table 4.3 summarises the results of Western blots of the scFv clones tested. The results show that the mouse R2 scFv was detected by the immune sera both with and without the human *c-myc* detection tag. The mouse anti- HEL his₆ (pOPE 90) and anti- HEL gene III (pSEX 20) scFvs that lack the human *c-myc* tail also stained positive with all mouse immune sera. Note that the mouse pre-immune sera did not cross-react with *E. coli* proteins (Figure 4.9). Therefore, there should also be no natural mouse antibodies that cross react with the gene 3 protein.

ScFV	V _H	V _K	Linker	Detection Tag	Mouse Test Bleed			9E10
					R2	R3	B4	
R2	mo V _H 2A Vgam3.8	moV _K 1- 117*01	G ₄ A (G ₄ S) ₂	<i>c-myc</i>	+	+	+	+
R2 minus <i>c-myc</i>	mo V _H 2A Vgam3.8	moV _K 1- 117*01	G ₄ A (G ₄ S) ₂	None	+	+	+	-
mo α HEL <i>c-myc</i> (pOPE 40)	mo V _H IB	mo V _K V	α-tub	<i>c-myc</i>	-	+/-	+	+
mo α HEL his6 (pOPE 90)	mo V _H IB	mo V _K V	α-tub	his ₆	+	+	+	ND
mo α HEL gene3 (pSEX 20)	mo V _H IB	mo V _K V	α-tub	gene III	+	+	+	
mo α HEL <i>c-myc</i>/his₆	mo V _H IB	mo V _K V	α-tub	<i>c-myc</i> /his ₆	+	+	+	

Table 4.3: Summary of the results of Western blots of scFv clones to test the *c-myc* detection tag as a potential epitope location. The results show that the *c-myc* tail may be a candidate but not the only region responsible for the immunogenic properties of scFvs.

ScFv	V _H	V _κ	Linker	Domain orientation	Mouse Test Bleed			9E10
					R2	R3	B4	
B3	hu V _H 1 DP-10	hu V _κ 2 DPK15	G ₄ A (G ₄ S) ₂	HL	+	+	+	+
B3 TB	hu V _H 1 DP-10 hu	hu V _κ 2 DPK15	AS	LH	-	-	-	+
R3	mo V _H 2A Vgam3.8	hu V _κ 1 DPK9	G ₄ A (G ₄ S) ₂	HL	+	+	+	+
R3DB	mo V _H 2A Vgam3.8	hu V _κ 1 DPK9	G ₃ AS	LH	+	+	-	+
R4	mo V _H 2A Vgam3.8	hu V _κ 1 DPK9	(G ₄ S) ₃	LH	+	+	-	+
3A11	mo V _H 2A Vgam3.8	huV _κ 6 DPK26	G ₄ A (G ₄ S) ₂	HL	+	+	+	+
3A11 DB	mo V _H 2A Vgam3.8	huV _κ 6 DPK26	ASG ₃	LH	+	+	+	+
3A11TB	mo V _H 2A Vgam3.8	huV _κ 6 DPK26	AS	LH	+	+	+	+
4A4	mo V _H 2A Vgam3.8	huV _κ 4 DPK24	G ₄ A (G ₄ S) ₂	HL	+	+	+	+
4A4TB	mo V _H Vgam3.8	huV _κ 4 DPK24	AS	HL	+	+	+	+
OKT3	mo V _H 2 gene H30	mo V _κ 4	(G ₄ S) ₃	HL	+	+	+	ND
OKT3DB	mo V _H 2 gene H30	mo V _κ 4	G ₄ S	HL	+	+	+	ND

Table 4.4: Summary of the results of Western blots of scFvs differing in linker length and domain orientation.

4.3.15 Detection of Epitope in the Linker Region of scFv

Diabody (DB) and tribody (TB) versions of the scFv clones were tested to determine whether an epitope was located in the linker region of the scFv. Analysis by Western blot was limited to denatured or linear epitopes only.

The V domain orientation of the multimer clones with shortened linkers tested had also been swapped from the common V_H-V_κ orientation to V_κ-V_H. This showed that the linker itself and its juxtaposition to V domain derived sequences in itself do not create an immunogenic B cell neoepitope on scFv (see discussion). The V_H FR4-I linker and V_κ FR4-I c-myc junction create good candidates for T cell neoepitopes but

not the linker-V κ FR-I junction. The results summarised in Table 4.4 show that B3 tested positive with all the sera whereas B3TB was negative.

4.3.16 Detection of a potential Epitope after Linker modification

The scFvs tested varied in the combination of linkers and detection tails (summarised in Table 4.5). R2 and mouse anti-HEL *c-myc* (pOPE 40) have mouse V_H and V κ domains and vary mainly in the linker. This is similar to the data obtained with B3 vs B3TB, but opposite to the 3A11 series. These results could indicate a role of the his₆ tail. However, other results exclude the his₆ tail, e.g. B3TB (Table 4.4) and the 4A4 ELISA (Figure 4.12) but not the Western blot (Table 4.4).

ScFv	V _H	V κ	Linker	Tail	Mouse Test Bleed		
					R2	R3	B4
R2	mo V _H 2A Vgam3.8	moV κ 1- 117*01	G ₄ A (G ₄ S) ₂	<i>c-myc his₆</i>	+	+	+
mo α HEL his ₆ (pOPE 90)	mo V _H IB	mo V κ V	α -tub	<i>His₆</i>	+	+	+
mo α HEL gene3 (pSEX 20)	mo V _H IB	mo V κ V	α -tub	gene 3	+	+	+
OKT3	mo V _H 2 gene H30	mo V κ 4	(G ₄ S) ₃	<i>c-myc his₆</i>	+	+	+
mo α HEL <i>cmyc</i> (pOPE 40)	moV _H IB	mo V κ V	α tubulin	<i>c-myc</i>	-	-	+/-

Table 4.5: Summary of the results of Western blots of scFvs with differing linkers and detection tails.

4.3.17 Detection of Epitope on scFv kappa constant domain fusion proteins

A series of scFvs with C κ domains replacing the *c-myc*/his₆ tail were tested in Western blot analysis to determine whether the *c-myc* tail was the potential epitope site. The results (Figure 4.23) showed that anti-B4 serum reacted with R3 human C κ fusion whereas R3 mouse C κ fusion protein was positive with anti-R3 serum. Other C κ fusions were positive for both B4 and R3 mouse antisera or none. Therefore, if there was an immunogenic epitope on the *c-myc*/his₆ tail, this cannot be the only epitope location.

ScFv	V _H	V _K	Linker	Mouse Test Bleed			9E10
				R2	R3	B4	
B3 c-myc his₆	Hu V _H I DP-10	Hu V _K II DPK15	G ₄ A (G ₄ S) ₂	+	+	+	+
B3 moCκ	Hu V _H I DP-10	Hu V _K II DPK15	G ₄ A (G ₄ S) ₂	ND	+	+	-
B4 huCκ	Hu V _H III B28e	Hu V _K VI DPK26	G ₄ A (G ₄ S) ₂	ND	-	-	-
R3 c-myc his₆	mo V _H IIA Vgam3.8	Hu V _K I DPK9	G ₄ A (G ₄ S) ₂	+	+	+	+
R3huCκ 210-2	mo V _H IIA Vgam3.8	Hu V _K I DPK9	G ₄ A (G ₄ S) ₂	-	-	+	-
R3moCκ 261-5	mo V _H IIA Vgam3.8	Hu V _K I DPK9	G ₄ A (G ₄ S) ₂	-	+	-	-
OKT3 cys c-myc his₆	mo V _H IIA gene H30	mo V _K IV	(G ₄ S) ₃	ND	+	+	+
OKT3 hu Cκ	mo V _H II gene H30	mo V _K IV	(G ₄ S) ₃	ND	-	-	-

Table 4.6: Summary of the results of Western blots of scFvs replacing the c-myc tail with Cκ.

4.4 DISCUSSION

A number of attempts have been made to make antibodies and scFvs immunogenic in order to elicit an increased immune response. These studies include making scFvs immunogenic using protein toxins derived from a microbial, plant and human sources conjugated with monoclonal antibodies. These toxins can be engineered directly into the antibody constant regions. Examples utilising this strategy include *Pseudomonas* exotoxin, which when conjugated to anti-CD22 has been shown to dramatically increase cell killing (Mansfield *et al.*, 1997). Examples of plant toxins include deglycosylated ricin a-chain conjugated with CD19 to confer a large increase in antibody-mediated cytotoxicity of malignant B-cells (Conry *et al.*, 1995). An alternative is the use of cytotoxic human protein Angiogenin, a human RNase, to induce apoptosis when delivered into the cytoplasm. Bacterially expressed CD30L–angiogenin fusion protein has been shown to elicit killing of CD30+ Hodgkin-derived cell lines (Huhn *et al.*, 2001). Fusion proteins to make scFvs immunogenic are discussed in greater detail in Chapter 1 Section 1.3.4.1- 1.3.4.3.

Another approach is the use of small toxic molecules usually DNA-complexing agents or inhibitors of the cell cycle. In this scenario the antibody conjugate is internalized and the toxic drug liberated after cleavage of a pH or enzyme-sensitive linker. As mAbs can target chemotherapy exclusively to cancer cells, more potent chemotherapy can be used when attached to mAbs than when administered systemically, for example maytansine conjugates (Smith, 2001). These small toxic drug molecules are advantageous as they have negligible immunogenicity and are relatively easy to handle in comparison with radionuclides. Mylotarg is a humanised anti-CD33 linked to calicheamicin for acute myelogenous leukaemia and was the first toxin conjugate to be approved. Further details can be found in Chapter 1 Section 1.3.1. In this chapter, we investigated the immunogenic potential of R3 scFv and the RAFT3 ZZ fusion protein.

4.4.1 Immunisation of CBA mice

Initial immunisations were carried out using R3 scFv and R3ZZ fusion protein with Complete Freund's Adjuvant (FCA) or adjuvant without immunogen. Some mice in all groups (including adjuvant only) developed abnormally large distended abdomens. Despite of this the mice did not exhibit any other symptoms.

Post mortems were carried out on all the mice and the organs were removed and stained by H&E. The mice had 2-10 ml ascites fluid (~5 ml average) in the abdomen at the time of the post mortem. This was cloudy, yellow and odourless. The ascites was tested by ELISA and did contain antibodies raised against the immunogens (data not shown). The large distended abdomen is a side effect attributed to FCA as these results were obtained in mice immunised with adjuvant only, i.e. adjuvant in the absence of the scFvs and the fusion protein. Tung *et al.* (1976) used this ascites for antibody production. We also observed a strong immune reaction against non-scFv *E. coli* proteins in Western blot. Bausinger *et al.* (2002) found an 'adjuvant effect' of *E. coli* endotoxin contaminating *E. coli* derived recombinant HSP70 preparations. Therefore, it is possible that *E. coli* proteins present at low levels in the scFv preparations enhance the apparent immunogenicity of scFvs. Some single chain Fvs can exhibit a pronounced tendency to form aggregates (Tan *et al.*, 1998). Although this was not studied in detail in the present study, it has been shown that protein aggregation can increase the immunogenicity of proteins (Schellekens *et al.*, 2002). The immunogenic effect of antibody aggregates can be eliminated by aggregate removal through ultracentrifugation prior to injection (Isaacs, 2001).

The mice were kept under special pathogen free conditions and could have been exposed to non-pathogenic *E. coli* prior to immunisation. The lack of reactivity of the pre-immune sera in Western blot could imply a role of contaminating *E. coli* proteins in the scFv preparations (Figure 4.39). It is possible that these impurities could have an adjuvant effect.

Freund's Complete Adjuvant (FCA) consists of mineral oil and mycobacterial components. The mineral oil acts as an antigen depot and the mycobacterial cell wall components act as a non-specific immune mediator. FCA is a potent adjuvant commonly used in animal research, as it can augment both humoral and cellular immune responses to a wide range of antigens. However, it is too toxic for use in humans and is now discouraged or banned by many institutional animal ethics committees due to its noxious side effects (Weeratna *et al.*, 2000).

There are other adjuvants available that are comparable or superior to complete Freund's adjuvant, but without apparent toxicity (Hartmann *et al.*, 2000). Other adjuvants that are currently used in animal research include Titermax Gold, monophosphoryl lipid and hepatitis B surface antigen (McCluskie & Weeratna, 2001). Due to limitations imposed by the project license we were unable to replace the adjuvant during the course of this study. Amendments have since been made to enable the use of other adjuvants.

The literature reviewed suggested that the most suitable adjuvant to replace FCA would be CpG DNA. CpG containing oligos have been shown to represent a powerful adjuvant for both humoral and cellular immune responses (Lipford *et al.*, 1997). Enhancement of humoral responses appears to be due to the strong synergy between the direct activation of B cells by CpG DNA and the signals, derived through the B cell antigen receptor, for both B cell proliferation and Ig secretion (Krieg *et al.*, 1995). In addition, antigen-specific humoral responses are likely to be enhanced by the induction of cytokines that could have indirect effects on B cells via T helper cells. Early studies suggested that CpG DNA induces a T_H1 like pattern of cytokine production dominated by IL-12 and IFN γ , with little secretion of T_H2 cytokines (Klinman *et al.*, 1996). However, subsequent studies showed that CpG could also stimulate a potent antibody response that depends on the DNA sequence context and species (Verthelyi & Klinnman, 2003). A CpG based adjuvant for antibody production in mice is commercially available (Immune Easy Mouse Adjuvant, Quiagen). CpG could affect the immunogenicity of scFvs quite differently from Freund's adjuvant. ScFvs without adjuvant could also be tested *in vivo* to mimic the situation in patients more closely.

4.4.2 Histological Analysis

The organs from immunised mice were harvested and stained with H&E. No significant changes in tissue morphology were noted. Therefore it was concluded that RAFT3ZZ does not induce any significant side effects as a result of repeated administration.

4.4.3 Detection of Antibodies Raised Against Proteins

The serum obtained from immunised mice was then tested in ELISA to detect antibodies raised against proteins. All 5 mice from each group were tested and gave similar results within the groups. Antibodies were raised against both R3 and R3ZZ. Furthermore, the results showed significantly better binding of sera from R3 immunised mice in comparison with R3ZZ immunised mice. This finding was unexpected and contrary to a study by Léonetti *et al.* (1998) that showed antigens fused to protein A or ZZ are substantially more immunogenic than the unfused antigens (Löwenadler *et al.*, 1987; Ducancel *et al.*, 1989; Löwenadler *et al.*, 1990). Leonetti, *et al.* showed that protein A or ZZ fusion and immunoglobulin protein A fusion facilitates antigen uptake by FcR positive antigen presenter cells (Leonetti *et al.*, 1998; Leonetti *et al.*, 1999).

The similarity of scFv-mediated melanoma binding by RAFT3 immune serum with the results of direct ELISA on plates coated with pure RAFT3 (Figure 4.7 and 4.8) suggest that serum cross-reactivity with contaminating *E.coli* proteins can only be a minor component (if any) in the direct ELISA assay. By contrast our data showed that scFvs are highly immunogenic and replacement of the human *c-myc*/his₆ tail by ZZ results in significant reduction of scFv immunogenicity in mice.

Since no anti-idiotypic response was observed, the immunogenicity of scFvs was investigated further instead. A further surprising result was that an optimal response was obtained following a single booster with R3 scFv compared to 4 boosters with R3ZZ. Mice immunised with the mouse R2 and human B4 scFvs showed an immune response similar to the mouse/human chimeric R3 scFv. This is a surprising result since up to 5 boosters can be required to obtain a good antibody response against poor immunogens or an anti-idiotypic response (Pervin *et al.*, 1997). Alfonso *et al.* (2002)

immunised melanoma patients repeatedly with a mouse Ab2 and the authors detected an Ab3 response after 3 to 4 booster. Titres against Fc of the Abs (HAMA) were 1:10,000 to 1:100,000. Typical anti-idiotypic titres were 1:200 to 1:3,200. Goldbaum *et al.* (1997) immunised mice with polyclonal but monospecific rabbit Ab2 (3 boosters). The best responding mouse had anti-rabbit IgG titre of 1:12,800. The anti-idiotypic titre of this mouse was 1:1,600. Zhou *et al.* (1995) immunised mice with polyclonal pig anti-PRV antibody (3 boosters). The anti-idiotypic titre was 1:81; and the anti-Fc titre was 1:10,000. McCormick *et al.* (1999) immunised mice with Mabs versus scFv and tested sera on Mab coated ELISA plates. The authors observed that mice are protected from subsequent tumour challenge and anti-idiotypic response. However, the ELISA itself would discriminate between anti-idiotypic and a response directed against non idiotype parts of the V domains.

Rajadhyaksha *et al.* (1995) also showed that three immunisations of 2F10 mAb induced a maximal immune response. These results indicate that R3 can elicit an immune response (1:25,000) on par with whole immunoglobulin and Fab fragments (Ferrone *et al.*, 1993).

No detailed studies have been reported that test the immunogenicity of scFvs in humans. Pavlinkova *et al.* (2000) tested the immunoreactivity of CC49 single-chain Fv antibody fragments using sera containing 'antiidiotypic' antibodies to mAb CC49 from patients treated with CC49 (mouse IgG) in clinical trials. The authors tested patient sera for reactivity with mouse CC49 scFv (m/m), humanised scFv (h/h) and a chain shuffled CC49 scFv with a mouse V_H and a human V_κ domain (m/h). The patient sera cross reacted with the mouse derived scFvs (m/m > m/h) but not with the humanised scFv. The study concluded that humanised scFvs should be less immunogenic in patients (Pavlinkova *et al.*, 2001). This seems likely but remains to be shown. In our opinion the data imply a cross-reactivity of patient sera with mouse framework encoded regions rather than an 'anti-idiotypic' response. Anti-idiotypic antibodies should also have cross-reacted with the humanised scFv.

ELISA was used to demonstrate that the anti-scFv antibodies were able to bind melanoma via R3 scFv using sera from R3 and R3ZZ immunised mice. Only sera from the R3 immunised mice were able to detect melanoma binding due to the stronger immune response as shown in Figure 4.10. It is possible that the apparently higher immune response of mice against R3 than against R3ZZ in cell ELISA and direct ELISA on pure scFv reflects the balance of mouse antibodies directed against the *c-myc*/his₆ tail and against other regions of scFv, such as the human V_κ. One has to assume that mice also make an immune response against the ZZ region of R3ZZ although this is technically difficult to test.

The results from these assays clearly demonstrate that the R3 scFv is mediating an immune response that is stronger than that against the R3ZZ fusion protein. This finding is contrary to the general belief that scFv fragments are weakly immunogenic (Kuus-Reichel *et al.* 1994) and could have very far-reaching implications for the future direction of developing these molecules for use in tumours. If there were an immune response in patients against scFvs the biodistribution of a diagnostic scFv (radio-imaging) would upon repeated administration become similar to R3ZZ (Chapter 5 Section 5.4.3). Furthermore, serum redirection of scFv to melanoma could have therapeutic effect. However, scFv complexes with IgG may not penetrate solid tumours efficiently and this could be a limitation of this approach. However, HMW-MAA is expressed on tumour neovasculature (Ruoslahti, 2002) and tumour penetration may not be necessary for a therapeutic effect.

However, immune responses against scFvs could also cause severe side effects, e.g. anaphylactic shock (LoBuglio *et al.*, 1988). An originally monomeric scFv could inadvertently cross-link its target antigen (via anti-scFv antibodies) and exhibit side effects (e.g. vascular leak syndrome caused by divalent OKT3 mAb (Norman *et al.*, 1993)). Therefore, particular emphasis was placed on confirming these results.

In order to test whether the immune response was directed against an epitope unique to R3 scFv sera binding to other scFvs was determined in ELISA. The scFvs R3, B4, R2 and 4A4 were initially tested. The results showed that the test sera were able to bind scFv R2, R3 and B4 but not 4A4 scFv (Figure 4.12). In contrast to the monomers R2, R3, B4 the 4A4 scFv is expressed as a mixture of monomers, dimers and trimers.

These initial results suggested that the epitope could be located at the bottom of the scFv fragments as these could be altered in scFv dimers and trimers (Perisic *et al.*, 1994; Pei *et al.*, 1997). The 3D structure of spontaneous multimers is unknown but could be similar to dia- or tribodies.

The immunogenicity of the parent monoclonal antibody, human IgG, B4 scFv, R2 scFv and the adjuvant alone was determined by immunising CBA mice. Mice were given 3 boosters as this seemed sufficient to elicit an immune response in the mice previously immunised with 5 boosters. The results (Figure 4.15) demonstrated that mouse anti-human IgG but not anti-LHM2 sera cross react with R3 scFv. Anti-R3 sera cross-reacted strongest, followed by anti-R2 sera. Anti sera against B4 and R3ZZ cross-reacted with R3 at similar levels (data not shown). The reactivity of anti-human IgG should be directed against R3 human V κ . The reactivity of anti-R2 should be directed against artificial parts of R3 (most likely the *c-myc*/his₆ tail). Artificial domains could be more immunogenic than the natural human kappa chain.

Testing for anti-idiotypic on whole cells or cell extracts showed no evidence for an anti-idiotypic response. This could have been due to a combination of low anti-idiotypic titre and high background binding of sera to cells (Figure 4.10). No positive control for competition or pure antigen was available. The high immunogenicity of R3 non-idiotypic regions could compete with an efficient anti-idiotypic response (immunodominance). Therefore, a more sensitive assay is required such as hybridoma formation and screening for rare anti-idiotypic clones. Anti scFv hybridomas could be tested for anti-idiotypic reactivity (e.g. binding to R3 but not B4). However, due to time constraints this possibility was not pursued.

4.4.4 Epitope Mapping

Initially ELISA was used to screen for possible epitope locations. After the completion of a few experiments testing the most likely regions, it was decided that ELISA would not be a suitable technique to map the epitope. This was primarily due to the limited volumes of immune sera. Western blot analysis was used as an alternative as it allowed screening of a larger number of clones with smaller amounts of immune sera. However, this limited the analysis to non-native, linear epitopes. On the other hand, denaturation could uncover epitopes concealed in native protein e.g. by domain interactions of scFv V domains. This may explain the low serum reactivity of the 4A4 scFv in direct ELISA (Figure 4.12) but high reactivity in Western blot (Figure 4.18).

Figure 4.9 illustrates the results obtained from Western blot analysis detecting a panel of scFvs with the R3 and R3ZZ pre and post immune sera. Although scFvs were detected, cross-reactivity with *E. coli* derived non-scFv proteins was high. This problem was overcome by making a pUC119 his₆ mock induction in parallel with the scFv inductions and analysis in parallel. In addition, incubation of the filters with the mock induction significantly reduced the binding of non-scFv proteins (Figure 4.18, b vs c). Unpurified scFv inductions were used for Western blot. Pre-immune sera did not bind the non-scFv proteins in Western blot. These *E. coli* proteins were not visible with 9E10 staining (Figure 4.18a) and were probably present at low concentrations in the scFv preparations used for immunisations. However, they were not detectable by Coomassie Brilliant blue staining of the purified proteins. It is therefore possible that low binding of immune sera to 'pure' scFv in direct ELISA was in part due to cross-reactivity with *E. coli* proteins present at low levels.

The initial results of the Western blots were unexpected as the 4A4 scFv that was bound by immune sera poorly in ELISA (Figure 4.12) was positive in Western blot (Figure 4.18). The bottom of the scFv is implied by the poor binding of sera to 4A4 in direct ELISA with pure and native scFv protein. Approximately 90% of protein bound to plastic becomes denatured (Butler *et al.*, 1993). It is not known how binding to plastic affects spontaneous multimers. In Western the scFv is denatured, i.e. a potential neo-epitope at the bottom of scFv should be disrupted unless it is a flexible

structure like the scFv linker (Zdanov *et al.*, 1994). Therefore, staining of R3 and R4 scFv in Western can only exclude the V_KFR4-linker-V_HFR1 junction as epitope location. The result implies the existence of more than one epitope as both B and T cell epitopes are required to induce an immune response against scFv. Furthermore, it is possible that the R3 and R2 scFvs are more immunogenic than the B4 scFv because they have a stronger functional T_H cell epitope. However, B4 must also have both types of epitopes otherwise an anti-B4 antibody response would not have been observed.

R2 is a mouse scFv and one has to assume that T_H cells specific for the R2 V domains are deleted or become anergic during mouse foetal development. Therefore, only the linker *c-myc* or his₆ tail or their artificial junctions with the V domains can trigger functional T_H cell response in mice. Epitope prediction for RAFT2 (<http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/Info.htm>) shows good T_H epitopes at the junctions of V_HFR4, V_KFR1 and V_KFR4 with artificial R2 sequences. The MHC anchor residues of these potential epitopes are conserved amino acids required to maintain the structure of β -strands at N and C termini of all V domains. All mouse scFvs with a (G₄S)₃ linker and *c-myc*/his₆ tail should have similar potential T_H neoepitopes at these junctions. The same prediction can be made for human scFvs and HLA-DR. The immunogenicity of R2 in mice implies the existence of proteasome cleavage site that generate functional MHC Class II binding peptides in CBA mice (H-2^k). Similar cleavage sites can also be predicted for humans using the PAPROC algorithm (Kuttler *et al.*, 2000; www.paproc.de).

The linker (*c-myc* or his₆) is flexible and is disordered in crystal structures of scFv and therefore an ideal candidate for the location of functional proteasome cleavage sites. Most scFvs could be good immunogens, even in the same species due to artificial structures. If this was the case then it could be possible to make scFvs suitable for radio-imaging by using dsFv derived from the same species and with no detection tag, i.e. no artificial sequences and using protein G for detection and purification. The absence of T_H neoepitopes should make such dsFvs non-immunogenic.

Other approaches for making antibodies non-immunogenic include preventing the recognition of murine immunogenic peptides from the antibody variable region by

human lymphocytes. This is achieved by site directed mutagenesis to make them human and/or to break the amphipathic motifs of the putative immunogenic epitopes in the variable region. *In vivo* studies have shown the 'detope' R3 antibody to be less immunogenic than its chimeric analogue (Mateo et al., 2000). This method has general applicability to reduce immunogenicity of chimeric antibodies possibly with therapeutic potential (Roque-Navarro *et al.*, 2003).

The panel of scFvs were analysed in Western blot to determine the most likely location(s) of the epitope(s) on the scFv structure. The bottom of the scFv initially seemed to be the most likely candidate region. This was because in the monoclonal antibody and the Fab structure this region is covered by the interaction with the C κ and C $H1$ domains. In the scFv this region becomes exposed and could trigger an immune response. The DB and TB clones have a shortened linker and a V κ -V H domain orientation whereas the R2, R3 and B4 scFvs have a V H -V κ domain orientation. Therefore, a potential epitope on the linker or adjacent sequences should be disrupted in the DB and TB clones. Therefore, this region seems an unlikely location for the B cell epitope.

B3, R3, 3A11 and 4A4 diabody and tribody scFvs were tested to determine the role of the linker and V domain orientation. Table 4.4 summarises the findings of Western blots with these scFvs stained with R2, R3 and B4 immune sera. The bottom of scFv as an epitope location was implied by the failure of pure 4A4 scFv to bind immune sera (thereby also excluding the *c-myc/his₆* tail) (Figure 4.12) and scFv mediated binding of sera to melanoma cells (excluding a location at or close to the antigen binding site of scFv) (Figure 4.10). However, the bottom of scFv is a native structure and could not be tested in Western blot. Therefore, the analysis focused on artificial and human parts of scFv as possible epitope locations.

There was no significant difference in the detection of monomer, diabody or tribody with the R2 and R3 immune sera. However, the B4 immune sera did not detect the B3TB and R3BD. Furthermore, it also did not detect the R4 scFv, which is a domain swapped antibody. However, there was no difference in the staining patterns for the 3A11, 3A11DB and 3A11TB. Similar results were found for the 4A4 and 4A4TB scFvs. It was therefore concluded that if the linker region was a candidate for the

epitope it could not be the only epitope location. One method to determine whether the scFvs share an epitope at the bottom of the scFv would be to construct a Fab fragment and use this as an immunogen. Due to time constraints it was not possible to test this, but remains an important question to be answered.

The linker was further tested using clones with an alternative linker (Tables 4.1 and 4.3). No significant difference in staining patterns was observed with scFvs with different linkers, i.e. polyglycine serine linker or the yeast α -tubulin linker. Therefore, the linker region is excluded as a possible site for the B cell epitope. These results agree with the diabody/ tribody and domain swap scFvs.

The results with scFvs without a *c-myc*/*his*₆ tail were less clear (Tables 4.5 and 4.6). The scFv human C κ fusion clones were almost not detectable by the R3 and B4 immune sera, whereas the scFv mouse C κ fusion clones were detectable. It is possible that there is an epitope on the V domain that could cross-react with mouse but not human C κ domains. Sequence comparison did not suggest the existence of such an epitope (data not shown). Other results (binding of immune sera to mouse anti-HEL scFv gene III fusion in Western but not to 4A4 in direct ELISA) raise further doubts about the *c-myc*/*his*₆ tail as a location for the immunodominant epitope on scFv. On the other hand, mouse antibodies were used as positive controls for *c-myc* and *his*₆ detection. Therefore, these sequences have to be immunogenic in principle in mice. It is possible that this immunogenicity could be occluded by an immunodominant epitope located on other parts of the scFv.

In summary, it seems unlikely that the complexity of these results can be explained by the presence of a single B cell epitope at any one region except the linker. The existence of more than one epitope appears more likely. Nevertheless, the data show that scFvs (irrespective of their origin- mouse, human or chimeric) are highly immunogenic in mice, and therefore one has to assume that scFvs could also be immunogenic in humans. This could interfere with the repeated diagnostic use of scFvs for radioimaging, but also suggests novel uses of scFvs for cancer therapy.

The results from the various epitope mapping assays and experiments to confirm the immunogenicity of scFvs indicate that there is more than one B cell epitope predominantly located on the V_κ, the *c-myc*/his₆ tail or its junction. There could also be a neoepitope for T helper cells generated during genetic engineering that stimulates the B cell response at least against the mouse R2 scFv. The exact location of these epitopes will require further studies by protein engineering and generation of anti-scFv hybridomas.

Chapter 5

Therapeutic scFvs

5 THERAPEUTIC SCFVS

5.1 INTRODUCTION

5.1.1 Immunotherapy

Whilst there appears to be tremendous potential for vaccines there has also been significant interest in immunotherapy for melanoma for over 50 years. However, to date no large prospective randomised trial has shown a survival benefit (Sabel and Sondak, 2002).

For many years, the field of immunotherapy of malignant diseases was divided into two distinct and at times antagonistic camps. One camp emphasising the importance of the cellular and the other the humoral effector arm of the immune system for destruction of tumour cells. In the following years appreciation of the complementary nature of T cell and antibody based immunotherapy stimulated interest in developing approaches that combine their advantages and minimise their limitations (Abken *et al.*, 1998). Furthermore, the realisation that tumour cells utilise multiple mechanisms to escape from immune recognition and destruction has stimulated interest in developing and applying immunotherapeutic strategies that target both humoral and cellular immunity to malignant cells (Wang *et al.*, 2000b).

5.1.2 T cell Activation and Retargeting

A widely used approach for immunotherapy is T cell activation and retargeting. This strategy uses a foreign substance to activate T cells and retargets antibodies against the tumour cell. Porter *et al.* (1997) used staphylococcal enterotoxin B for T cell activation and retargeting with antitumour x anti-CD3 F(ab)₂ bispecific antibodies. The authors suggested that this approach can be successful not only to cure mice but also to provide protective immunity against targeted and non-targeted tumour antigens. This approach has been shown to generate host anti-tumour immune responses and to prevent the recurrence of pulmonary metastases (Rice *et al.*, 1999).

5.1.3 Antibody Dependent Cell Mediated Cytotoxicity

It has been proposed that inhibitory receptors modulate the *in vivo* cytotoxic response against tumour targets of both antibody dependent and independent pathways (Bolland *et al.*, 1999). Antibody Dependent Cell Mediated Cytotoxicity (ADCC) had been shown to be an effective mechanism for *in vitro* and *in vivo* tumour cytotoxicity. The mechanism of ADCC is discussed in detail in Chapter 3 section 3.1.5.

Recombinant fusion proteins consisting of the extracellular domains of immunoregulatory proteins and the constant domain of IgG are a novel class of human therapeutics that mediate antibody dependent cellular cytotoxicity (Taylor *et al.*, 2002). Jasinska and co-workers (2003) demonstrated the strong inhibiting effects on tumour growth *in vitro* and *in vivo* of monoclonal antibody Trastuzumab by ADCC. This mechanism has been used in numerous studies on ADCC mediated cancer therapy *in vivo* (Kawase *et al.*, 1985; Munn *et al.*, 1991; Clynes *et al.*, 2000).

5.1.4 Complement Mediated Cytotoxicity

Complement mediated cytotoxicity (CDC) is the activation of the complement system against tumour cells. Antibody bound to the target cell surface fixes complement, resulting in the formation of the membrane attack complex that punches holes in the target cell membrane and subsequent cell lysis. This mechanism is discussed in greater detail in chapter 3 section 3.1.7.

5.1.5 Therapeutic scFvs

ScFvs consist of the V_L and V_H and represent the minimal antigen binding region of antibodies. They were originally developed to reduce the HAMA response observed with whole monoclonal antibodies in patients. The smaller size of scFvs enables increased tumour penetration and confers advantages in pharmacokinetics and biodistribution.

Immunoconjugates composed of an anti-melanoma scFv fused to the Fc region of human IgG₁ as the effector domain, mediated specific *in vitro* lysis of human melanoma cells by natural killer cells and complement (Wang *et al.*, 1999b). Other

examples include the use of scFvs with genetic fusions such as scHLA-A2 complexes containing immunodominant tumour or viral specific peptides (Lev *et al.*, 2004). Kikuchi *et al.* (2004) developed a single-chain scFv derived from a murine monoclonal antibody, MABL, which specifically bound to human CD47 and induced apoptosis of leukemia cells.

5.1.5.1 Bispecific Antibodies

A combination of bispecific antibodies against CD3 and CD28 has shown tumour growth inhibition (Katayose *et al.*, 1996). The same antibodies have been used as adoptive immunotherapy for bile duct carcinoma in a mouse model (Kodama *et al.*, 2002). The authors demonstrated the potential of three bispecific antibodies, against CD2, CD3 and CD28, acting in synergy to maximise the therapeutic effect. Recombinant bispecific scFvs specific for CTLs have also been shown to cure Non-Hodgkin's lymphoma in an *in vivo* model (De Jonge *et al.*, 1998). Bruenke *et al.* (2004) demonstrated the use of recombinant bispecific scFvs directed against FcγRIII (CD16) and human leucocyte antigen (HLA) class II for the treatment of primary cells from B cell lymphoma patients.

5.1.5.2 ScFv against Human Insulin like Growth Factor

Li *et al.* (2000b) investigated the mitogenic and anti-apoptotic activities of an anti-IGF-IR scFv antibody (Insulin Growth Factor I Receptor). The authors expressed the scFv as fusion to human IgG₁ Fc. The single chain Fv antibody caused an inhibition of tumour growth of MCF-7 breast cancer cells after 13 days of treatment both *in vitro* and in athymic mice. The authors concluded that the therapeutic effect was due to the inhibition of IGF-IR function and apoptosis triggered by scFv-Fc binding.

5.1.6 Radioimmunotherapy using dimeric scFvs

Single chain Fv fragments have also been used for radio immunotherapy. Pavlinkova *et al.* (1999) used a dimeric scFv construct for the radio immunotherapy of colon cancer in a human xenograft mouse model. The authors found a 60% survival of mice 150 days post therapy with ¹³¹I labelled CC49 (scFv)₂. However, 75% survival was

achieved with labelled CC49, the parental monoclonal antibody (IgG), administered for the same duration and at a 3-fold lower dose.

5.1.7 Radioimmunotherapy Using Tetrameric ScFvs

The use of divalent sc(Fv)_2 and tetravalent $[\text{sc(Fv)}_2]_2$ CC49 scFv fragments for radio immunotherapy was investigated by Goel *et al.* (2001). Treatment with tetravalent scFv in athymic mice bearing human colon carcinoma xenografts showed a statistically significant prolonged survival with both single and fractionated administrations (Goel *et al.*, 2001).

5.1.8 Immunotoxin Therapy

Flavell *et al.* (2001) demonstrated the therapeutic effects of two immunotoxins in SCID mice. Anti-CD7 immunotoxin (IT) HB2-SAPORIN and anti-CD38 IT OKT10-SAPORIN were administered i.v. The authors found a significant delay in time to leukaemia development. In contrast, the unmodified HB2 and OKT10 antibodies (murine IgG₁ antibodies) exerted relatively weak therapeutic effects. Having tested these in combination therapy the authors observed that the combination of the two immunotoxins led to the greatest therapeutic efficacy in comparison with the individual immunotoxins and antibodies. This study clearly shows that the therapeutic potency of immunotoxins greatly outweighs those of antibodies alone. The therapeutic mechanism was a combination of direct killing by Saporin and ADCC.

5.1.9 Preclinical Drug Development

It has been estimated that about 800 of the new agents in development today are at a preclinical stage and approximately 500 are being tested in phase 1, 2 or 3 trials (Mariani *et al.*, 2003). Between 1975 and 1994, a total of 29 of the 280 drugs in development were approved by the FDA, which yields an approximately 10% rate of approval. Clinical development and approval of 20 drugs in the 1990s took on average 10.8 years, with an expense of \$800 million US dollars per drug (Mariani *et al.*, 2003).

Many new drugs are only active in subsets of patients and show a 10% or lower response rate, such as raf kinase inhibitors or IL-2. The development of new anticancer agents is slowed down by low success rates. Different factors are likely to be responsible for this low efficiency. These include *in vitro* models that may be inaccurate, species differences in ligand metabolism and/or pharmacokinetics (e.g. a higher clearance in a mouse model may lead to a higher tolerance of an agent) or variations in tissue sensitivity (Chabner *et al.*, 2003). Furthermore, human tumours exhibit a far higher degree of diversity and mutability than experimental tumours (Mariani *et al.*, 2003).

5.1.10 *In Vivo* Models

Methods used to assess drug activity in humans often lack sensitivity. Responses to many of the new drugs in development cure cancer in mice but only reduce tumour growth rates in patients (Mariani *et al.*, 2003).

With the advantage of being genetically similar to humans, mice are small and relatively inexpensive to maintain and are therefore ideal candidates for *in vivo* studies. Their short life span and rapid reproductive rate make it possible to study disease processes in a living organism. Mice are used in biomedical research as models of human beings in order to understand the human body, determine the effects of drugs and develop treatments for diseases. When one compares the mouse and human genomes there are many similarities but also many differences. For example, mice are inbred whereas humans are outbred. Furthermore, SCID and nude mice often used for cancer research are immunoincompetent and have obvious limitations as models for immunotherapy.

The use of animals as models for human disease has proved to be indispensable in understanding the causes and biology of cancer. Animal models play a crucial role in the preclinical testing of new anti-cancer drugs and treatments. Mice, rats and hamsters are the most commonly used animals for modelling human tumours (Teicher, 2002).

The advantages of using rodents in medical research are that their physiology and genetics are well understood. They are relatively cheap and easy to maintain. In addition, mice tend to develop cancer with relative ease in response to chemical carcinogens. The tumours develop over months rather than years, as is the case in larger animals. Tumour cells that have been transplanted from one rodent to another go on to form complete tumours in immunodeficient rodents (Amundadottir *et al.*, 1996; Lipkin, 1997; Rosenberg *et al.*, 1999; Rovigatti *et al.*, 1998).

5.1.10.1 Origins Of Mouse Genetics

The use of mice as the leading model system for medical research can be traced as far back as to the start of human civilisation, when mice became communal with human settlements (Morse *et al.*, 1981). Spontaneously arising coat-colour mutants were observed and recorded (including ancient Chinese references to dominant-spotting, waltzing, albino and yellow mice). By the 17th century, mouse dedicatives in Japan and China had domesticated many varieties as pets. Europeans subsequently imported favourites and bred them with local mice (thereby creating progenitors of modern laboratory mice as hybrids among *M. m. domesticus*, *M. m. musculus* and other subspecies). In Victorian England, 'fancy' mice were prized and traded, and the first National Mouse Club was founded in 1895 (Keeler, 1931).

The rediscovery of Mendel's laws of inheritance in 1900 led to pioneers of the new science of genetics recognising that the discontinuous variation of fancy mice was akin to that of Mendel's peas, and therefore they set out to test the new theories of inheritance in mice. They established mating programmes and created inbred strains, resulting in many of the modern, well-known strains, e.g. C57BL/6J (Morse, 1978).

Genetic mapping in the mouse began with Haldane's report (Haldane *et al.*, 1915) of linkage between the pink-eye mutation and albino loci in a linkage group that was eventually assigned to mouse chromosome 7.

Originating from a common ancestral genome approximately 75 million years ago, the mouse and human genomes have been shuffled by chromosomal rearrangements. Therefore, it is possible to recognise chromosomal regions in the two species that have descended relatively intact from a common ancestor (Waterston *et al.*, 2002).

5.1.10.2 Transgenic Mice

A transgenic mouse is a mouse with a gene from another animal and is produced by introducing the foreign gene into the egg that develops into the mouse (Brinster *et al.*, 1981). The transferred gene becomes a permanent part of the animal's genetic makeup. This allows studying the influence of a particular gene on tumour development. Transgenic mice have been produced that develop particular tumours or that are particularly sensitive to cancer-causing viruses and chemicals. Transgenic mice are valuable for testing suspected carcinogens, new cancer treatments, and new measures to prevent cancer (Hansen and Khanna, 2004).

5.1.10.3 Nude mice

The nude mutant was described in 1966 in a closed stock of mice in a laboratory in Glasgow, Scotland (Flanagan, 1966). The absence of the thymus in nude mice was first discovered and described in 1968 (Pantelouris, 1968). The nude mouse has been used abundantly to study cancer. Its immunodeficient status allows human tumour xenografts to be grafted onto the mouse without rejection. This allows the study of specific human cancers and the subsequent testing of new treatments and diagnostic methods (Kang *et al.*, 1999; Pavlinkova *et al.*, 2001; Kelland *et al.*, 2004).

The main characteristics of the Balb/c nude mouse include the absence of the thymus and hair due to impaired keratinisation resulting in breakage of the hairs within the follicles (Pritchard *et al.*, 1974). The failure of the thymus development is generally believed to be due to some basic defects in the embryonic ectoderm where the first detectable abnormalities in the development of the nude mouse thymus occur during the formation of a cervical vesicle on day 11 of its embryonic life (Cordier *et al.*, 1980). A normal complement of B-lymphocytes is found in these mice. The lymphocyte population is almost entirely composed of B-lymphocytes, and a relatively normal IgM response to thymus-independent antigens has been observed. However, only a poor response to thymus-dependent antigens can be found (Pritchard *et al.*, 1974).

Balb/c nude mice have a small population of T cells. The antibody response is confined to IgM. The response to T-cell dependent antigens is low, resulting in a

compensatory increase in the level of natural killer cells (NK). A small number of lymphocytes with T-cell antigens such as CD5, CD8 and Thy-1 on the cell surface can be identified. The presence of a small number of lymphocytes with T-cell markers may result from direct passage from the mother/euthymic littermates or develop under the influence of humoral factors from the mother/euthymic littermates. However, it is possible that early steps in T-cell differentiation, to the point of TCR expression, can occur in athymic mouse bone marrow (Benveniste *et al.*, 1990; Palacios *et al.*, 1991). Although the athymic nude mouse is largely deficient in peripheral T cells, the number of lymphocytes bearing T-cell markers (CD4, CD8) and the $\alpha\beta$ or $\gamma\delta$ T-cell receptor (TCR) increases steadily with age (leakiness of the nude phenotype).

Palacios *et al.* (1991) demonstrated that the spleen is one of the extrathymic sites where T-cell progenitors can rearrange TCR γ and TCR δ genes. However, no evidence for TCR β gene rearrangements was found in this organ. Furthermore, the extrathymic TCR gene rearrangements appear to be distinct and less diverse than those found in the developing thymocytes (Palacios *et al.*, 1991; Kennedy *et al.*, 1992).

Lymphokine activated killer cells (LAK) and natural killer cells (NK) are more frequent in nude mice than in normal mice and their activity seems enhanced compared to euthymic mice (Hasui *et al.*, 1989; Møller Nielsen & Heron 1984). The activity of NK cells is reduced in BALB/c-*nu* mice after transfer of T cells (both CD4⁺ and CD8⁺), indicating a regulatory relationship between NK cells and T cells (Harada *et al.*, 1989).

Mononuclear cells are also present and macrophage function seems to be enhanced in nude mice (Cheers *et al.*, 1975). The number of mast cells appears to be normal in the skin and lymphatic tissues (Wlodarski *et al.*, 1982).

The ability to grow xenografts is the basis for the widespread use of Balb/c nude mice as hosts for transplanted human tumours and therapeutic studies on human tumours (Povlsen *et al.*, 1974; Spang-Thomsen *et al.*, 1977; Fogh *et al.*, 1978; Fogh *et al.*, 1982; Engelholm *et al.*, 1987).

Early studies revealed that Balb/c nude mice were useful for serial transplantation of human solid tumours. The morphological and functional characteristics of the tumours were maintained during serial transplantation (Merenda *et al.*, 1975). The Balb/c nude mouse has a relatively high take rate for tumours compared to other nude mouse strains or immune deficient mice (Dagnaes-Hansen *et al.*, 1991). Maruo *et al.* (1982) found that the tumour growth rate of human gastric cancers was lower in Balb/c nude mice compared to CBA, NSF and NIH nude mice. In addition, the T cell deficiency can make the nude mouse a valuable tool in basic immunologic studies (Pritchard *et al.*, 1974; Kindred *et al.*, 1978). The drug action and possible side effects can be studied in nude mice without interference from the immune system. Nude mice are also more sensitive to transplacental exposure to carcinogens (to induce skin tumours) than their *nu*⁺ littermates (Anderson *et al.*, 1982).

5.1.10.4 SCID mice

The SCID (severe combined immunodeficiency) mutation was discovered during tests of the immune response of CB-17 mice. Dr. Bosma discovered the strain in 1980 during routine laboratory tests on the immune system in mice (Bosma *et al.*, 1983). The first SCID mice were an accident of nature, the product of chance matings of apparently normal mice that carried a recessive mutant gene now called *scid*. Some of the offspring inherited a complete pair of *scid* alleles and were born with the *scid* defect. The *scid* mutation has since been transferred to various genetic backgrounds. Homozygous SCID mice have a functional haematopoietic system, natural killer cells, granulocytes and monocytes/macrophages but no mature B or T cells (Blamkert *et al.*, 2002). The mice are severely immuno-compromised and at high risk of infections. SCID mice are 2-3 times more radiosensitive than Balb/c mice and show an increased skin reaction after irradiation. The radio sensitivity of SCID mice depends on their genetic background (Biedermann *et al.*, 1991). SCID mice have been used extensively as a mouse model with human tumour xenografts. However, the SCID mutation is leaky in 2-15% of mice older than 12 weeks of age, depending on genetic background and housing conditions (Nonoyama *et al.*, 1993). By the age of about 12 months virtually all SCID mice are leaky. This leads to an oligoclonal B and T cell expansion. Such leaky mice will reject xenografts. In addition, 10 to 15% of old SCID mice will develop thymic T cell lymphomas (Bosma *et al.*, 1988).

5.1.10.5 Humanised SCID mice

SCID mice accept human lymphocyte xenografts and can be used as a tumour immunotherapy model with a semi functional human immune system (Mule *et al.*, 1992). The humanised SCID mouse is often used as a model for T cell targeting against human tumours and infectious diseases. A main limitation of this mouse model is a host versus graft (HvG) response of mice against the human lymphocytes and conversely a graft versus host (GvH) response of the human lymphocytes against the mouse (Dorshkind *et al.*, 1985). The HvG response may result in variability of the lymphocyte xenograft take and functionality from mouse to mouse. The GvH response can lead to a fatal wasting disease characterised by progressive weight loss, paleness and ruffled fur (Santini *et al.*, 1995).

The usefulness of human SCID mice as a valid model depends on the minimisation of both HvG and GvH responses. This has been achieved with antibody treatment to eliminate host natural killer (NK) cells (Murphy *et al.*, 1992; Somasundaram *et al.*, 1995), granulocytes (Lozupone *et al.*, 2000; Santini *et al.*, 1998) or monocytes/macrophages (Shibata *et al.*, 1998; Fraser *et al.*, 1995). However, the effect of this is transient and mice treated e.g. with anti asialo-GM1 antibody will recover their NK cell population within about 9 days (Barry *et al.*, 1991). Gamma or X-ray irradiation with or without antibody treatment has also been used to reduce the HvG response. However, SCID mice are very radiosensitive and irradiation may increase leakiness of the SCID phenotype and promote the development of murine thymomas (Shiptz *et al.*, 1994; Murphy *et al.*, 1994).

Keeping mice in isolated ventilated cages, use of laminar flow hoods or antibiotics in the drinking water may control some of these effects. Subcutaneous implantation of human lymphocytes minimises the GvH response. Long-term engraftment of human lymphocytes is best achieved by intraperitoneal injection but this increases the GvH response (Iwanuma *et al.*, 1997). The latter can be blocked by administration of neutralising antibodies against human CD40 or its ligand CD154. However, this leads to abnormalities of human B cell and T cell function and may increase the susceptibility of mice to infectious diseases (Chen *et al.*, 1995; Lazarus *et al.*, 1999). As an alternative to minimise the GvH response engraftment with lymphocytes or

lymphocyte subsets that have been pre-stimulated with tumour cells *in vitro* have been used (Schmidt-Wolf *et al.*, 1991; Cesano *et al.*, 1994).

5.1.10.6 CBA Mice

Strong developed this mouse strain in 1920 from a cross between a Bagg albino female and a DBA male. The strain CBA was selected for a low mammary tumour incidence. It is now widely distributed and used as a general-purpose strain. It has a low lymphocyte anti-phytohaemagglutinin response (Heiniger *et al.*, 1975). A good immune response to low doses of bovine gamma-globulin (Levine *et al.*, 1970) and a good splenic immune response to pneumococcal polysaccharide have been reported for some but not all CBA strains (Amsbaugh *et al.*, 1972). CBA mice make a good immune response to ovomucoid but a poor response to ovalbumin (Vaz *et al.*, 1971a; 1971b). The mice respond to the synthetic polypeptide Glu₅₄, Lys₃₆, Ala₁₀ but not to Glu₅₇, Lys₃₈, Ala₅ (Pinchuck *et al.*, 1965). A low immune response to gangliosides (Kawashima *et al.*, 1992) and a high natural killer cell response to the immunostimulant 7-allyl-8-oxoguanosine have also been observed (Pope *et al.*, 1994).

5.1.11 Metastasis

Metastasis formation is responsible for most cancer deaths (Chambers *et al.*, 2001). Metastasis is the spread or movement of cancer cells from the primary cancer site to another area of the body. This requires anchorage independent growth. Most normal cells with the exception of certain white blood cells do not have this ability. During metastasis formation tumour cells penetrate the fibrous boundaries that normally separate one tissue from another. The tumour can also infiltrate the walls of blood or lymph vessels and shed cancer cells into the circulation. These tumour cells are carried downstream to become lodged in the next capillary bed (Nicolson, 1988). The circulation to the liver also carries tumour cells shed from the cancer where secondary tumours often arise. Similarly, tumour cells from other areas of the body can be carried by the blood through the heart and on to the lungs, where they start metastatic lung tumours (Fidler, 2002). The main steps of metastasis formation are illustrated in Figure 5.1.

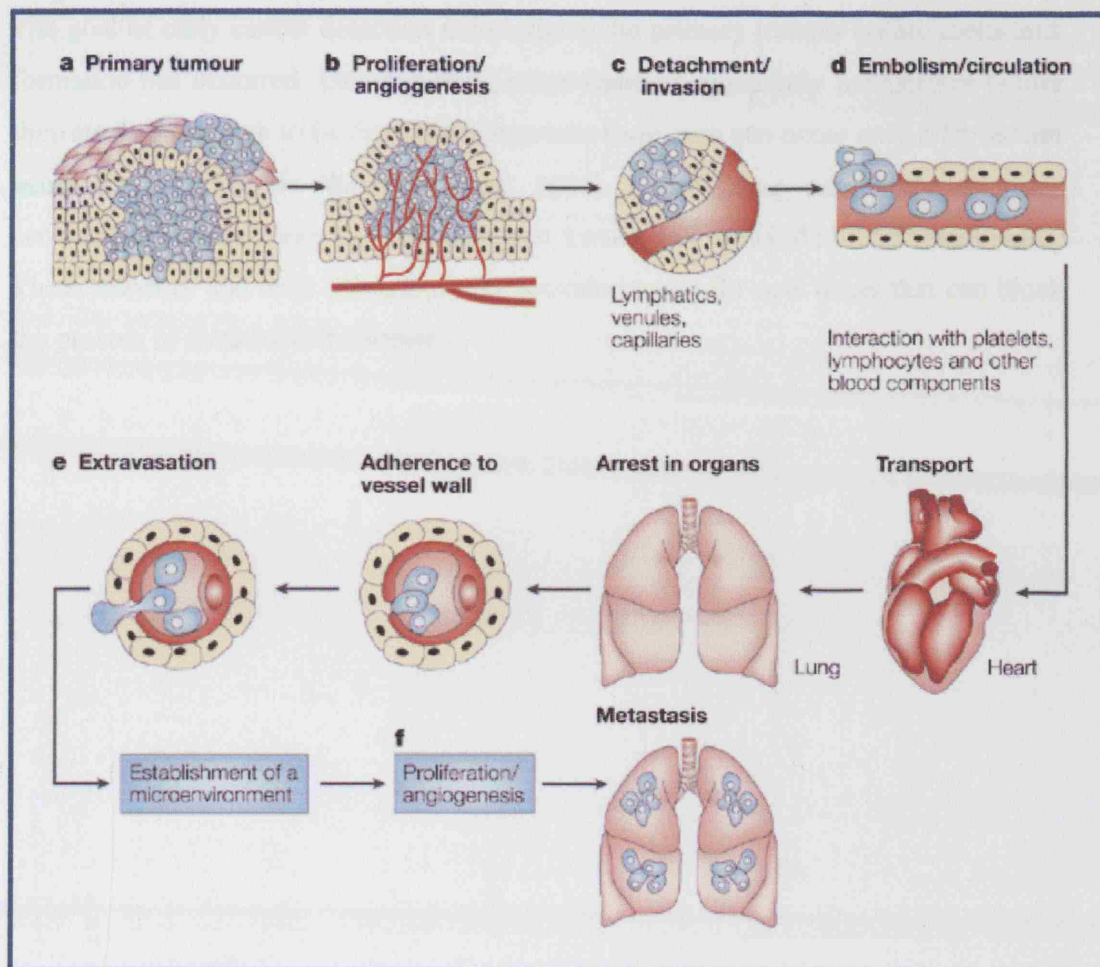


Figure 5.1. The main steps in metastasis formation (Fidler, 2003).

Tumour cells shed into the lymph system often establish themselves in the nearest cluster of lymph nodes (the draining lymph nodes), where they grow before spreading to more distant parts of the body. Fewer than 1 in 10,000 cells shed from the primary tumour are thought to survive, but these are enough to spawn secondary tumours elsewhere in the body (<http://www.acs.ohio-state.edu/units/cancer/handbook/metastas.pdf>). About 30 percent of patients with newly diagnosed solid tumours have detectable metastases. About half the remaining patients will be cured by their initial treatment. The remainder will have undetectable metastases that will eventually develop into tumours. Tumour staging includes determining whether a malignancy has spread beyond the primary tumour. This is a major factor in determining a patient's prognosis (Balch *et al.*, 2003).

The goal of early cancer detection is to remove the primary tumour before metastasis formation has occurred. Unfortunately, some tumours apparently metastasize before they are large enough to be detected. Metastasis formation can occur only after certain genes are switched on (Khanna *et al.*, 2001). These genes encode the enzymes necessary for cancer cells to penetrate other tissues and to invade blood vessel walls. These enzymes and their substrates may provide targets for new drugs that can block the process of metastasis formation.

5.1.12 Hypothesis

Following the analysis of RAFT3ZZ *in vitro* (Chapter 3) and the immunogenicity of single chain Fv fragments (Chapter 4) we tested the therapeutic potential of antibodies in a living model. Balb/c nude mice are not an ideal model due to the lack of T cells and a non-functional B cell response. Nevertheless, nude mice have all necessary immune components to mediate CDC and ADCC, the mechanisms of melanoma kill by RAFT3ZZ *in vitro*. Furthermore, the therapeutic potential of immunogenic scFvs can be tested in the Balb/c nude mouse model by co-administration of a mouse anti-scFv mAb.

On the other hand, the CBA mouse model should allow testing of the potential side effects of antibodies due to non-specific activation of the immune system.

5.1.13 Aims:

The aims of the work described in this chapter were to:

- Test the therapeutic efficacy of the scFv protein A fusion molecule in a mouse model, thus evaluating its activity in a living model.
- Analyse the side effects of repeated administration on immune organs.
- Investigate different routes of administration for repeated doses (i.e. tail vein vs intraperitoneal)
- Investigate the possibility of using scFv as a form of vaccine therapy.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of A375M Cells for Tumour Inoculation

A375M tumour cells were used for all animal experiments. Cells were cultured as described in Chapter 2 Section 2.5.1. Cells were harvested from confluent flasks under sterile conditions at room temperature within 1 hour before inoculation of mice.

5.2.2 Tumour Inoculation

Immuno-deficient female Balb/c nu mice (6-8 weeks) were bred from existing animal stocks maintained at the Gray Laboratories. The mice were housed in MB1 plastic cages (North Kent Plastics) holding a maximum of 20 mice and fed on a diet of standard expanded pellets (B & K Universal). They were implanted with A375M tumour cells when they reached between 6 to 8 weeks of age. Five mice were used per data point.

Standard precautions were observed when handling immuno-compromised animals. Hands and forearms were scrubbed with antiseptic agents before touching the animals. All clothing except underwear was removed before entering the animal house and surgical hats, masks, dedicated (clean) overalls, latex gloves and operating boots were worn at all times.

A clean operative field was established by laying tissue paper onto the bench and spraying this with 70% ethanol. The cell suspension was drawn up into a 1 ml syringe (NHS supplies) with a 23 G needle (Microlance). The mice were anaesthetised in anaesthetic jars containing tissue soaked with Enflurane™. A subcutaneous injection of $\approx 100 \mu\text{l}$ of the cell suspension (1×10^7 cells in PBS) was made into the right flank of each mouse. Tumours were allowed to grow until they reached a maximum of 13 mm mean geometric diameter before the mice were culled.

5.2.3 Investigation of Tumour Take

Tumour take experiments were carried out by testing the effect of different numbers of tumour cells for inoculation on subsequent tumour growth. A375M cells were cultured (Section 2.5.1) and harvested. Viable melanoma cells were counted using a haemocytometer and trypan blue staining. Different tumour loads (1×10^5 , 1×10^6 , 1×10^7 cells) were made up to 100 μ l PBS per mouse and inoculated s.c. into the right flank of Balb/c nude mice (see section 5.2.2).

5.2.4 Single Dose of Therapy at Time of Tumour Inoculation

Viable melanoma cells were counted using a haemocytometer and trypan blue staining. 1×10^7 human melanoma cells were made up to 100 μ l per mouse and inoculated s.c. into the right flank of Balb/c nude mice. Ten μ g protein was administered with the cells at the time of tumour inoculation. Fifty μ g LHM2 or 9E10 mAb were administered as their size is approximately five times larger than scFv. The tumour volume was measured with callipers. Tumour volume was calculated using the formula described by Forouhi *et al.* (1994) as shown in Figure 5.1. This formula was chosen based on the results of a study by Tomayko *et al.* (1989). The authors compared the volumes, areas and diameters predicted by a range of formulas with the actual weights of 50 tumours ranging from 0.46 to 22.0 g established in nude mice as xenografts of human cell lines. In addition to determining how well each formula predicted relative tumour size they analysed how well each formula estimated actual tumour mass. The ellipsoid volume formulas ($\pi/6 \times L \times W \times H$ and $1/2 \times L \times W \times H$) were the most accurate for estimating tumour volume.

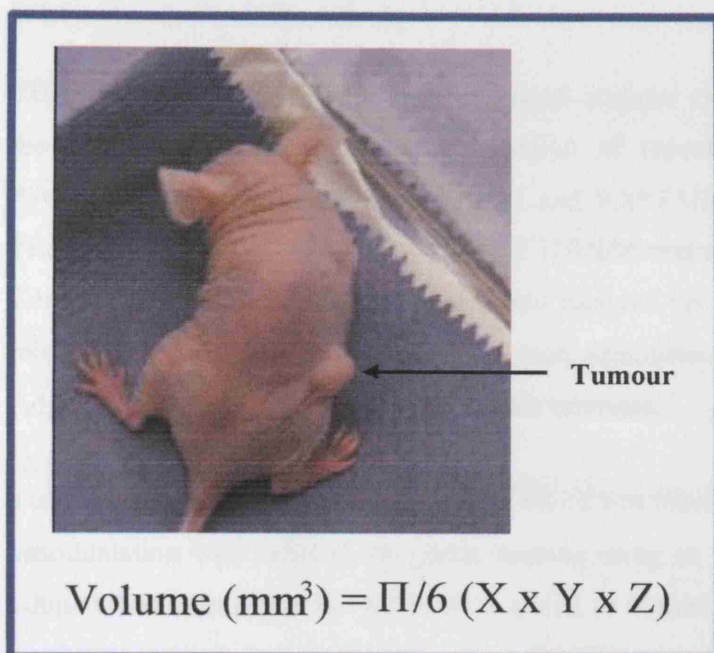


Figure 5.1: Balb/c nude mouse 18 days following a subcutaneous injection with A375M melanoma cells into the right flank. The tumour illustrated measures 7mm (+/-1 mm). The tumour dimensions (L, W, H) were used to calculate the tumour volume.

5.2.5 Repeated Administration i.v.

Mice were treated with 50µg antibody and/ or 10µg R3 scFv at the time of tumour inoculation (Section 5.2.4). Repeated doses were administered at days 3 and 5 via tail vein injection. Tail vein vasodilatation was induced by gentle heating using an infrared lamp and protein in 100µl PBS was injected. Mice were returned to their cages after they had recovered from the anaesthetic. They were monitored on a regular basis (3-4 times a week) and tumour volumes measured as described in section 5.2.4.

5.2.6 Repeated Administration i.p.

Mice were treated with 50µg antibody and/or 10µg R3 scFv at time of tumour inoculation (Section 5.2.4). Repeated doses of protein were administered at days 6, 11 and 16. Mice were returned to their cages. They were monitored on a regular basis (3-4 times a week) and tumour volumes measured as described in section 5.2.4.

5.2.7 Analysis of side effects

CBA mice aged 6-8 weeks with a functional immune system were used to determine the side effects induced by administration of repeated doses of 25µg RAFT3, RAFT3ZZ and adjuvant alone. RAFT3 and RAFT3ZZ were made up to 100µl in PBS pH7.2 (Gibco-BRL). The antibody solution was mixed thoroughly with 100µl Complete Freund's Adjuvant (Sigma) and injected i.p. into the right flank of CBA mice. Five boosters at the same dose were administered with Incomplete Freund's Adjuvant (Sigma) at approximately 14-day intervals.

Ten days post immunisation a sample of blood was taken from the tail vein. Tail vein vasodilatation was induced by gently heating using an infrared lamp, approximately 100µl blood was extracted and EDTA added to 50mM. The blood was centrifuged for 5min at 1000 rpm and analysed on RAFT3 coated 96 well microtitre plates to determine whether antibodies were raised against the immunogen. The results of these assays are described in detail in Chapter 4. After the completion of the boosters the spleen, liver and kidneys were harvested and stained by H&E.

5.2.8 Histological Analysis

Following the completion of the course of treatment with R3, R3ZZ and the adjuvant alone, mice were culled by cervical dislocation. A post mortem was performed on the mice and the spleen, kidney and liver removed for histological analysis. Figure 5.2 illustrates the location of the various organs.

Organs were harvested and rinsed with PBS and then fixed in 10% buffered formalin (a histological fixative) for 24 hours before being processed to wax overnight on a Tissue Tek VIP histological tissue processor. Following embedding in paraffin wax 4mm sections were cut, dried for 1hour at 60°C and stained with Haematoxylin and Eosin stain.

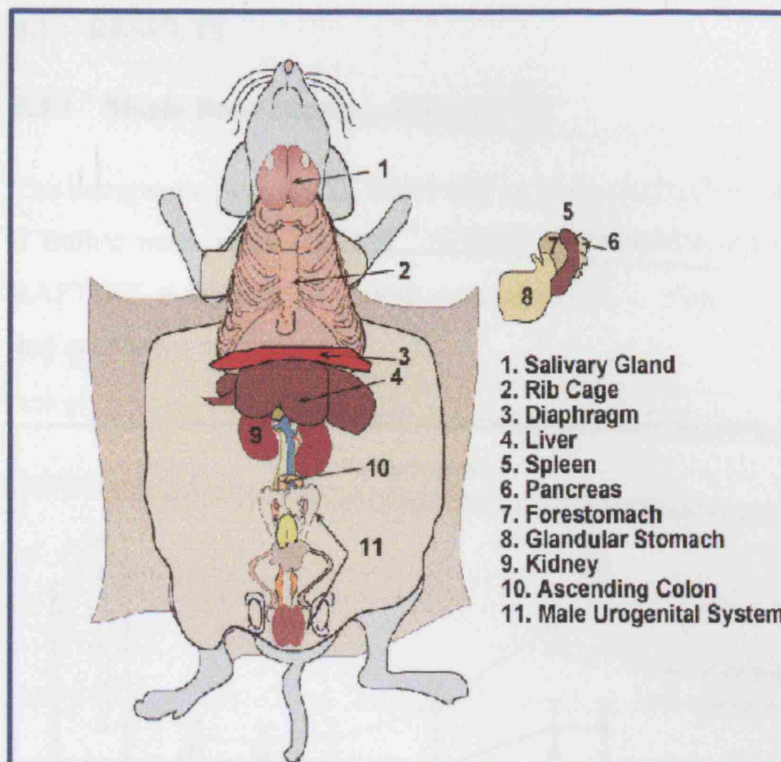


Figure 5.2: Illustration of the gross anatomy of the male mouse after gastrointestinal tract removal
 (<http://home.ncifcrf.gov/vetpath/intestine.html>).

5.3 RESULTS

5.3.1 Single Dose Therapy of RAFT3ZZ

The therapeutic potential of RAFT3ZZ as discussed in Chapter 3 was tested *in vivo* in a Balb/c nude mouse model. Suitably anaesthetised mice were inoculated with RAFT3ZZ at the time of tumour inoculation (s.c.). Tumour volumes were measured and are shown in Figure 5.3.

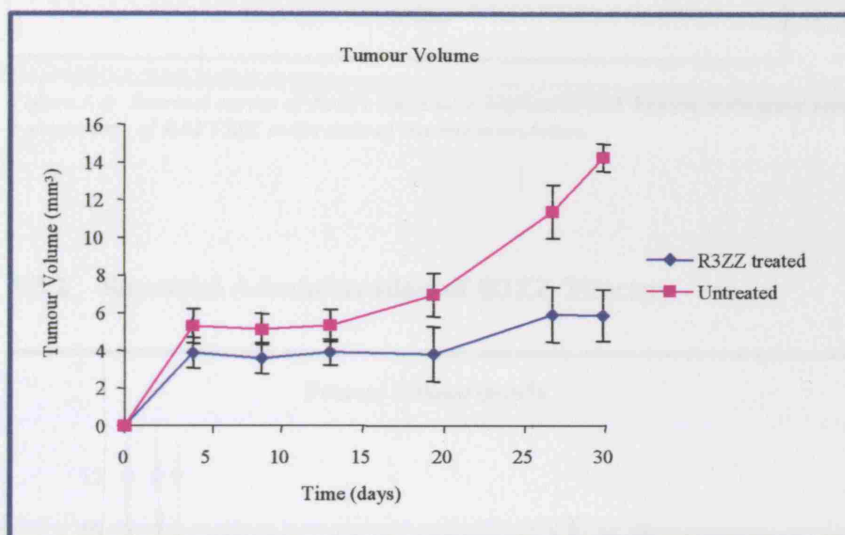


Figure 5.3: Tumour volume (mm^3) of human melanoma xenografts on Balb/c nude mice treated with a single dose of RAFT3ZZ at time of tumour inoculation (s.c.). Five mice were used for each group.

There was a significant reduction in tumour volume in the group of mice treated with a single dose of RAFT3ZZ compared with the control group inoculated with the melanoma xenografts in PBS. Mice were culled when their tumours reached the maximal permitted size (1.3mm mean diameter).

The survival curves for RAFT3ZZ treated mice and the untreated group are illustrated in Figure 5.4. The R3ZZ treated group had a 100% survival at 30 days. In comparison the untreated group had a 20% survival at 30 days.

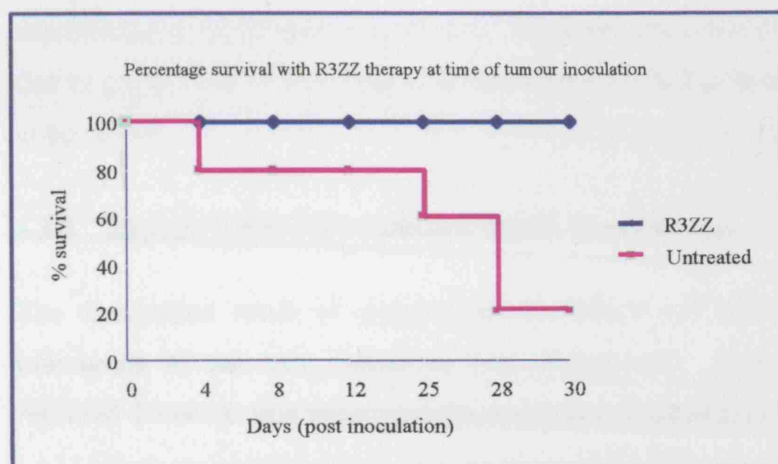


Figure 5.4: Survival curves of Balb/c nude mice implanted with human melanoma xenografts and treated with a single dose of RAFT3ZZ at the time of tumour inoculation.

5.3.2 Repeated Administration of R3ZZ Therapy - i.v.

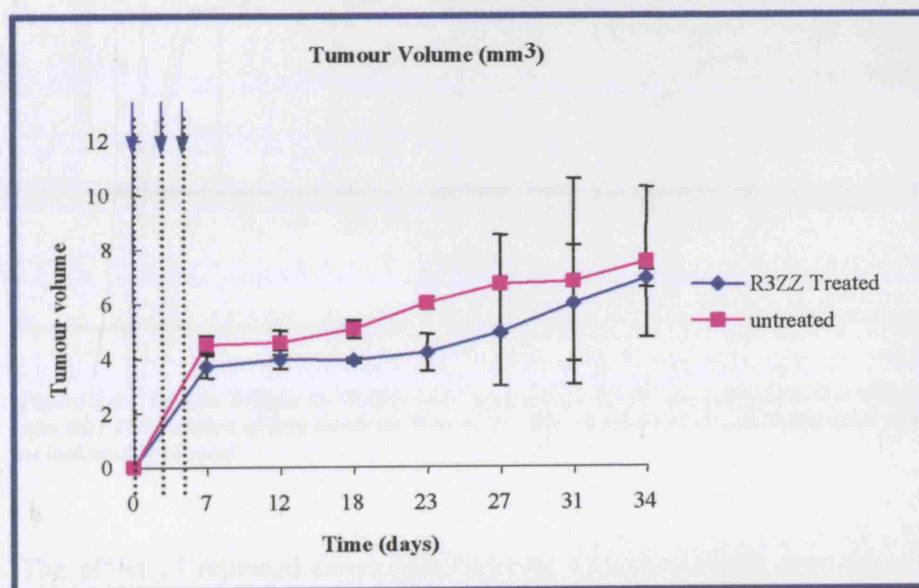


Figure 5.5: Tumour volume of Balb/c nude mice implanted with human melanoma xenografts and treated with RAFT3ZZ at time of tumour inoculation (s.c.). Repeated doses were administered i.v. at days 3 and 5 as indicated by arrows.

The therapeutic efficacy of RAFT3ZZ was tested further by administering repeated doses i.v. at days 3 and 5. The tumour volumes obtained are shown in Figure 5.5. Repeated doses of RAFT3ZZ did not improve the therapeutic efficacy but resulted in reduced tumour growth retardation when compared with the single dose

administration experiment (Figure 5.3). The large error bars observed at days 27-34 is due to group sizes of less than 5, as some tumours had grown large and the mice had to be culled.

5.3.3 Repeated Administration of R3ZZ Therapy - i.p.

The unexpected result of repeated administration i.v. could have been due to the inaccuracy of tail vein injections (see discussion). Therefore, administration of repeated doses i.p. was tested and the results are illustrated in Figure 5.6.

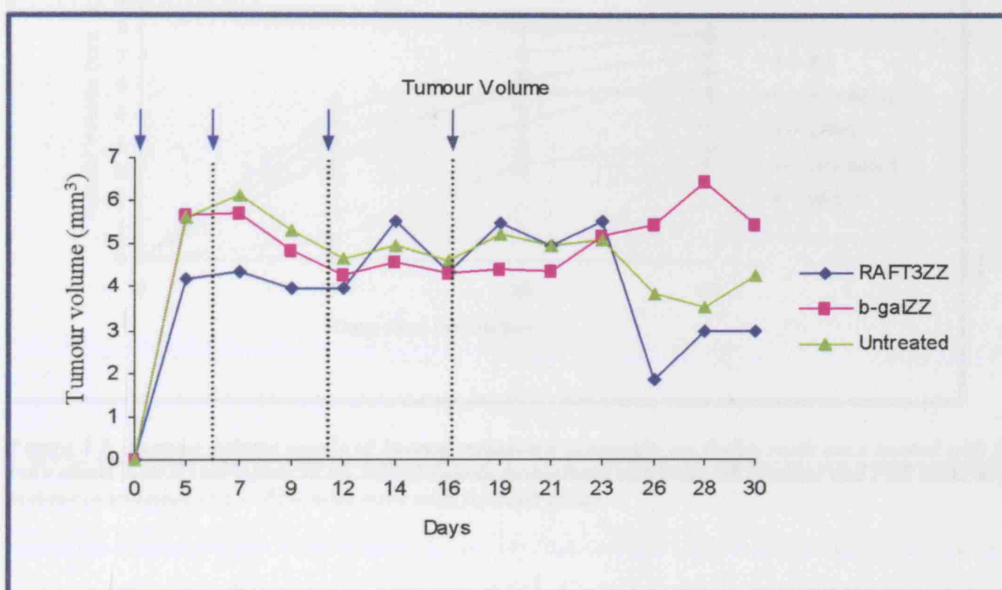


Figure 5.6: Tumour volume of Balb/c nude mice implanted with human melanoma xenografts and treated with RAFT3ZZ at time of tumour inoculation (s.c.). Repeated doses were administered i.p. at days 6, 11 and 16 as indicated by arrows.

The effect of repeated doses administered intra-peritoneally was also disappointing. There was no improvement in the treatment with RAFT3ZZ. The tumour volumes were similar to the untreated and β -gal ZZ treated groups.

5.3.4 Therapeutic Effect of RAFT3 scFv

The unexpected finding of the immunogenicity of RAFT3 scFv as described in detail in Chapter 4 was explored further to test whether its immunogenic properties would make the scFv suitable as a potential vaccine therapy.

Balb/c nude mice were inoculated with human melanoma cells and a single dose of the protein at the time of inoculation. R3 scFv alone, R3 + 9E10 (mouse anti-c-myc mAb; IgG₁), 9E10 alone, the parent whole monoclonal antibody LHM2 (mouse IgG₁) and PBS alone were tested for their therapeutic potential. The results are illustrated in Figure 5.7.

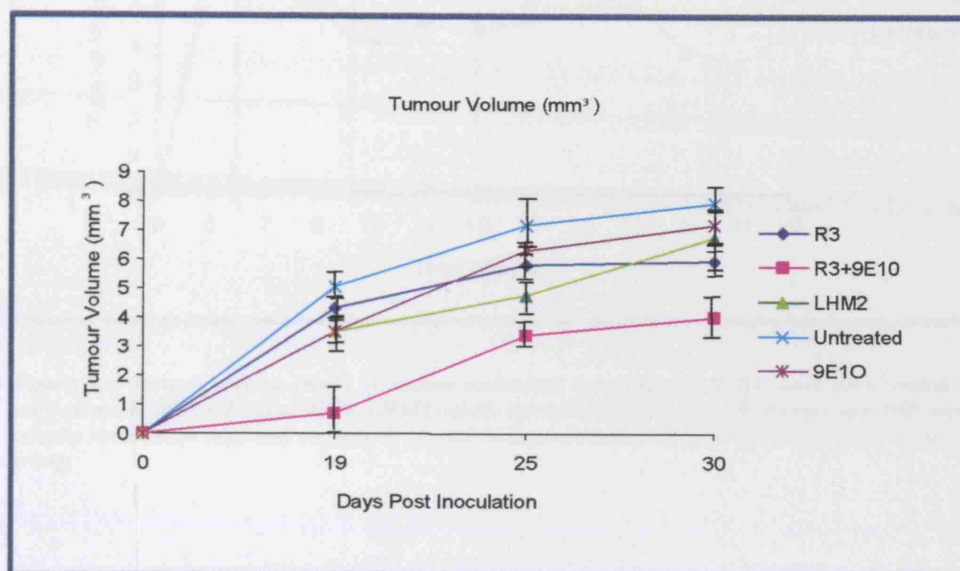


Figure 5.7: Tumour volume (mm³) of human melanoma xenografts on Balb/c nude mice treated with RAFT3 scFv alone, RAFT3 scFv plus 9E10, LHM2 (whole monoclonal antibody), 9E10 alone and PBS alone at time of tumour inoculation (s.c.). Five mice were used for each group.

Mice treated with RAFT3 scFv and 9E10 monoclonal antibody had significant tumour growth retardation in comparison with the negative control R3 scFv alone, 9E10 alone, LHM2 and the untreated group.

5.3.5 Repeated Administration of R3 scFv Therapy - i.p.

The therapeutic effect of repeated administration of RAFT3 scFv was determined by administering repeated doses i.p. at days 6, 11 and 16. The results are illustrated in Figure 5.8. Repeated administration of proteins i.p. had no therapeutic effect in comparison with the RAFT3 scFv alone and the untreated group.

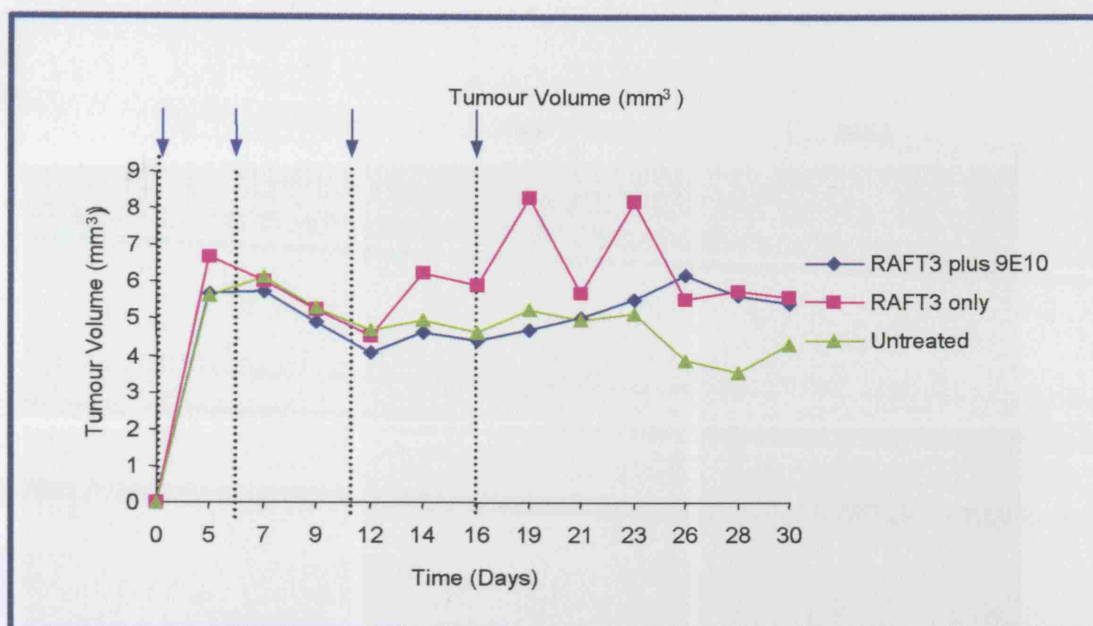


Figure 5.8: Tumour volume (mm³) of human melanoma xenografts on Balb/c nude mice treated with RAFT3 scFv alone, RAFT3 scFv plus 9E10, LHM2 (whole monoclonal antibody), 9E10 alone and PBS alone at time of tumour inoculation (s.c) and on days 6, 11 and 16 (i.p.) as indicated by arrows. Five mice were used for each group.

5.3.6 Analysis of Side Effects.

Previous work by Blau *et al.* (1997) has suggested that protein A leads to non-specific immune activation. In order to test for possible side effects of treatment with RAFT3ZZ mice that had been administered repeatedly with RAFT3ZZ, RAFT3 or adjuvant alone, as described in more detail in section 4.3.1, were examined for signs of immune activation. Post mortems did not reveal differences between the 3 groups of mice. Organs of interest were harvested and analysed by histology. The sections were fixed and stained with H&E. Figure 5.9 shows representative sections obtained from mice immunised with R3, R3ZZ in comparison with mock-immunised mice (adjuvant without antibody).

The sections show some effects as a result of immunisation. These include fluid accumulation in the kidney of R3ZZ treated mice. However, there were no significant differences in the morphology of the tissues. Therefore, there were few visible side effects as a result of repeated administration of R3ZZ.

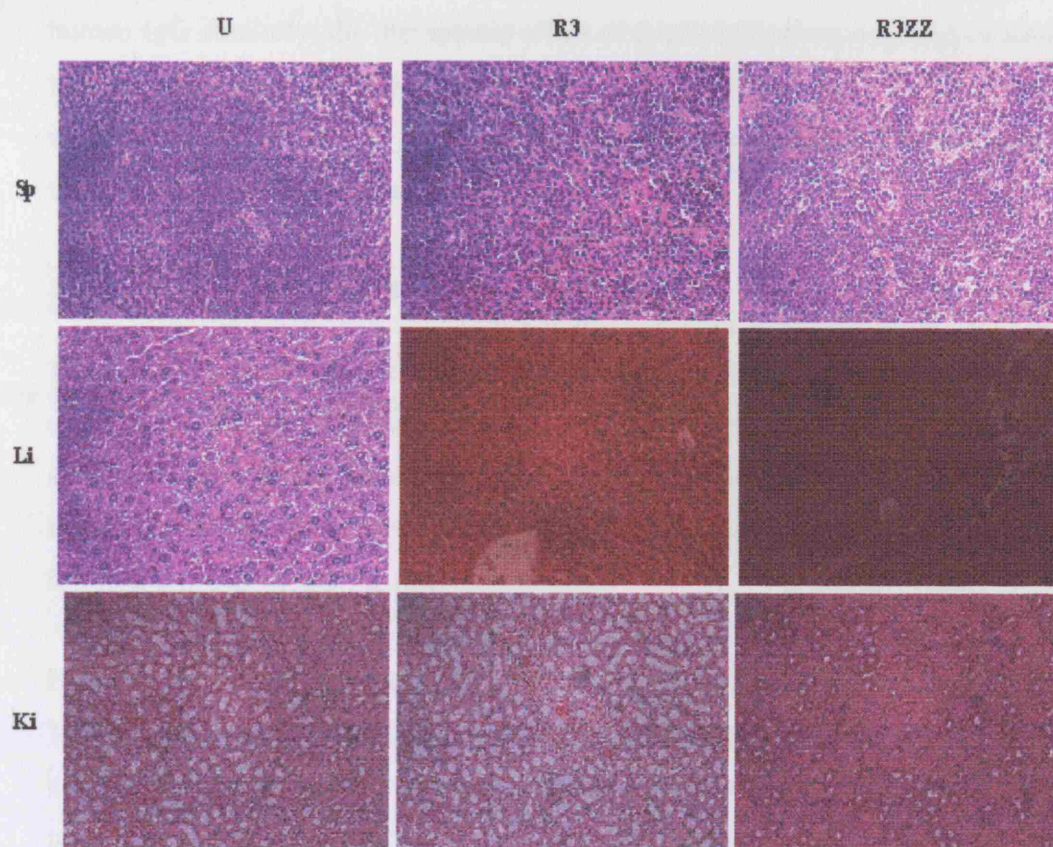


Figure 5.9: Histological sections of organs harvested following immunisations. Sections are representative of mice administered with adjuvant alone (u), R3 and R3ZZ. Spleen (top row), liver (middle row), and kidney (bottom row) were analysed

5.4 DISCUSSION

The *in vivo* model is a mouse model and since RAFT3ZZ interacts with mouse and human IgG similarly, the therapeutic effect of RAFT3ZZ in mice should be similar to that in humans allowing a relatively realistic extrapolation to humans. This is important as at present *in vivo* models especially for the active immunotherapy of melanoma remain unsatisfactory.

5.4.1 Therapeutic Potential of RAFT3ZZ

The therapeutic potential of RAFT3ZZ was investigated by administering a single dose at the time of tumour implantation (s.c.) and repeated doses i.v. and i.p. A significant therapeutic effect was observed with a single dose of therapy at the time of tumour inoculation. A growth delay was observed rather than prevention of tumour formation in this model for micrometastases.

However, in subsequent experiments using repeated doses R3ZZ was not as effective. The repeated administration experiment proved disappointing (Figure 5.5). Repeated doses i.v. initially resulted in a significantly slower tumour growth compared to the untreated group. However, after 27 days despite the treated group having smaller tumours there was no statistically significant difference between the treated and untreated groups. Repeated doses i.p. also failed to demonstrate any therapeutic effect.

Nude mice have lower IgG₁, IgG_{2a} levels and therefore different IgG isotype proportions compared with normal mice (Izui *et al.*, 1981). This affects clearance, e.g. if a mouse with a low IgG₁ level is treated with a therapeutic IgG₁ antibody, the therapeutic antibody will be cleared faster in Balb/c nude mice than in normal mouse strains. This could be one of the reasons for the observed differences. If the ZZ tail were cleaved *in vivo* the remaining R3 part would accumulate in the kidney leading to faster clearance. The biodistribution of R3ZZ was tested in nude mice with melanoma xenografts and was found to be very similar to LHM2 mAb for kidney, liver and blood at 48 hours (Odili, MD Thesis 2003). Therefore, R3ZZ is stable *in vivo* for 48 hours, with no evidence for increased liver or kidney clearance. However, this could be different in patients where R3ZZ can be expected to remain in circulation over a

much longer time period. Finally, R3ZZ with 2 IgG molecules bound will probably be cleared more effectively through the liver than LHM2 mAb. Therapy with R3ZZ could be improved by increasing the number of doses and by administration of higher amounts of protein.

5.4.2 Side Effects of R3ZZ

A number of studies demonstrated that the use of *Staphylococcus aureus* protein A induces inflammation *in vivo* (Blau *et al.*, 1997; Kremlev *et al.*, 1997). However, this is in contrast to the findings of Borron *et al.* (2000) who suggested an anti-inflammatory role of Protein A. The side effects observed with *Staphylococcus aureus* protein A have been attributed to contamination of preparations with lipopolysaccharide (LPS) (Wright *et al.*, 1997b; Wright *et al.*, 1999; Karkar *et al.*, 1997).

Furthermore, Subbulakshmi and colleagues (1998) demonstrated the potential use of protein A to reduce toxic effects of 3-azido-3-deoxy thymidine (AZT) for therapy in Swiss Albino mice. The authors demonstrated that pre-treatment of animals with protein A, biweekly for two weeks alleviated haematopoietic toxicity of AZT by increasing colony-forming units (erythroid) granulocyte monocyte. Increased levels of erythropoietin in the blood plasma and increased cellularity of spleen, thymus and bone marrow were also observed. This study supports the use of protein A in combination with other therapies to reduce side effects.

The histological sections of kidney, spleen and liver showed limited side effects when compared with untreated mice. The mice developed distended abdomens but this was attributed to Freund's adjuvant (Sato *et al.*, 1999; Sato *et al.*, 2004). It is also possible that the reduced size of ZZ may contribute to the reduction in side effects induced by protein A.

5.4.3 Pharmacokinetics

The pharmacokinetics of RAFT3, RAFT3ZZ and LHM2 (the common parental monoclonal antibody) were studied by J. Odili (MD thesis, 2003) in Balb/c nude mice. The results showed that RAFT3ZZ with a size similar to RAFT3 (39 kDa vs

28kDa) behaves more like a monoclonal antibody (150 kDa). RAFT3ZZ has a serum half-life similar to the monoclonal antibody suggesting serum retargeting and IgG recruitment *in vivo*. This shows that the antibody remains in circulation for longer and could mediate prolonged immune activation.

	Molecular Weight (kDa)	$t_{1/2 \alpha}$ (min)	$t_{1/2 \beta}$ (min)
R3	28	8	189
R3ZZ	39	48	337
LHM2	150	37	384

Table 5.1: Pharmacokinetics of RAFT3 scFv, RAFT3ZZ and the parent whole monoclonal antibody LHM2 as determined in nude mice (Odili, MD Thesis 2003).

The biodistribution data of RAFT3ZZ show that tumour localisation of RAFT3ZZ was not as high as with LHM2 Mab. Renal accumulation of RAFT3ZZ was significantly higher than with LHM2. Biodistribution in liver, spleen, lung, muscle and bone was similar for RAFT3ZZ and LHM2. The biodistribution of RAFT3ZZ is of minor importance for therapy, as the accumulation of the molecule in other organs such as the kidney should not affect its therapeutic efficacy. However, the somewhat inferior biodistribution of RAFT3ZZ compared with LHM2 could be indicative of instability of RAFT3ZZ *in vivo*, and this needs to be addressed.

Relatively low tumour: kidney ratios observed with RAFT3ZZ could be due to susceptibility to protease cleavage in tumour and kidney (Tas *et al.*, 1999). The use of scFvs that appear more stable *in vivo* could reduce renal accumulation. Addition of more than 2 Z domains could also increase the tumour to kidney ratio.

Single chain Fvs usually have a positive electric charge. Our laboratory has made scFvs with a neutral and mildly negative charge. These show reduced kidney accumulation, believed to be due to reduced electrostatic interaction with the negatively charged kidney membranes. Onda *et al.* (2001) showed that a scFv toxin

fusion protein modified to have a negative electric charge exhibits reduced liver cytotoxicity. The authors concluded that lowering the isoelectric point of the Fv may be one approach to reduce the nonspecific toxicity of recombinant immunotoxins and other Fv fusion proteins without loss of antitumour activity (Onda *et al.*, 2001). It is therefore possible that such scFvs with a negative charge fused to ZZ would accumulate to a lesser extent in the kidney.

5.4.4 Therapeutic Potential of RAFT3 scFv

The therapeutic potential of RAFT3 scFv was tested by co- administering the scFv with 9E10. 9E10 is a mouse monoclonal antibody (IgG₁) that recognises the *c-myc* peptide epitope, EQKLISEEDL (Hilpert *et al.*, 2001) of scFvs.

Balb/c nude mice do not have T cells but have FcR positive cells such as B cells, natural killer cells and macrophages as well as a functional complement system. For the purpose of serum IgG retargeting the Balb/c nude mouse model is therefore an immunocompetent model. The therapeutic effect of R3 plus 9E10 was similar to R3ZZ and remains unconvincing without further experiments.

5.4.5 Limitations of Mouse Models

Animal models show far higher response rates compared with humans who have a more variable and heterogeneous treatment response (Lewis, 1998; Mariani, 2003). Animal models have their limitations and results obtained through animal experiments must be extrapolated cautiously to humans. One of the major shortcomings of animal experiments is that extensive inbreeding has made many rodent strains genetically identical whereas humans, on the other hand, are genetically diverse. Thus, mice might respond very similarly in an experiment from one individual to another, whereas humans might respond to the same treatment with greater variability from one individual to another.

Animals and humans have important differences in their metabolic, physiological, and hormonal systems. For example, animals can differ greatly from humans in what carcinogens they metabolise and how they metabolise them. Typically the mouse metabolism is 12 times faster than that of humans (Dedrick *et al.*, 1992). The

differences that exist between different species of rodents can result in some substances causing cancer in rats, but not in mice or hamsters. An example of this is the controversy that arose when saccharin was linked to bladder cancer in 1977 (Howe *et al.*, 1977). It was claimed that the mechanism of cancer induction by saccharin in rats did not apply to humans. This was confirmed by subsequent studies (Whysner *et al.*, 1996; Zurlo *et al.*, 1998). Rats have been shown to have a 100 to 1,000 times higher concentration of globulin and albumin in their bladders than humans and it is the interaction of these two proteins with urine that leads to saccharin-induced bladder cancer (Swenberg *et al.*, 1989).

A limitation of using transgenic animals as cancer models is that cancer generally arises from a single tumour cell proliferating within a bed of normal tissue, overcoming the controls normally imposed by cell-cell interaction. In a transgenic model abnormal cells that carry the mutated gene surround the tumour cell. This difference could make the biology of these tumours different from that of spontaneous tumours. E.g. HGF/SF transgenic mice develop melanoma. However, these tumours are not UV induced and metastasise only rarely limiting the usefulness of this mouse model (Takayama *et al.*, 1997).

Finally, activity *in vitro* is not always an accurate indicator of the effects *in vivo* (Eccles, 2001). Patient's individual responses are determined by multiple factors including expression of the target antigen (e.g. signalling molecules that might compensate if the former is inactivated), levels of circulating Ig or immune complexes and the functional status of their immune cells. Therefore, the extrapolation of both *in vitro* data and the effects observed in murine models does not guarantee an effective therapy in patients. Hence, a combination of mechanisms may be the ideal treatment strategy where a number of immune responses are triggered to maximise the tumour kill.

5.4.6 Future Work

A number of different venues can be explored to improve the cytotoxic potential of both RAFT3ZZ and R3 scFv with 9E10. One example is the use of a cocktail of

scFvs. This would enable targeting of different epitopes on the tumour and may improve melanoma kill.

The use of multimeric scFvs (di-or tribody) with 9E10 or as a ZZ fusion could allow the recruitment of more IgG molecules and hence more potent tumour specific cytotoxicity.

5.4.6.1 Co-administration with antibodies

The co-administration of other proteins and CpG DNA could be investigated further. CpG containing oligonucleotides are a powerful adjuvant for both humoral and cellular immune responses (Lipford *et al.*, 1997) in various murine disease models. In addition, CpG is comparable or superior to complete Freund's Adjuvant but without apparent toxicity (Hartmann *et al.*, 2000).

Another possible direction for future study is to identify the lymphocyte population activated by R3ZZ and then co-administer stimulating cytokines or antibodies against appropriate lymphocyte surface activation markers. This could allow a more potent stimulation of the immune response and hence tumour specific cytotoxicity.

One possible avenue to be explored is the co-administration of β -glucan. The C3 receptors on human leukocytes do not trigger the killing of tumour coated with C3b and iC3b ligands. However, there is evidence to suggest that phagocytes and natural killer cells express CR3. In microorganisms β -glucan binds to the C-terminal lectin domain of CD11b and primes CR3 for cytotoxic degranulation (Vetvicka *et al.*, 1996; Ross, 2000). However, tumour cells lack β -glucan and therefore cannot trigger CR3-CDC. The administration of β -glucan intravenously in mice has shown to regress tumours and promote long-term survival (Yan *et al.*, 1999; Hong *et al.*, 2003). Therefore, co-administration of β -glucan and R3ZZ or R3+9E10 could improve complement mediated cytotoxicity considerably.

5.4.6.2 Decrease Complement Resistance *In Vivo*

The main problems associated with complement mediated strategies for therapy are the resistance of melanoma cells against attack by complement and a possible

undiscriminatory attack against normal cells (Junnikkala *et al.*, 1994). These problems could be overcome by co-treatment with an anti-CD59 antibody. Studies have shown that the level of CD59 regulates the extent of complement mediated cytotoxicity in human melanoma cells. Brasoveanu *et al.* (1995) demonstrated that CD59 was present in 8 of 12 melanoma cell lines and that its function may be to regulate the host-tumour interaction by protecting the neoplastic cells from complement lysis. The authors observed that masking CD59 with a monoclonal antibody increased lysis by homologous complement in a dose dependent manner. Staining of 38 benign and malignant lesions exhibited expression of CD59 consistently *in vivo* (Brasoveanu *et al.*, 1996). Therefore, methods for increasing the efficacy of complement lysis of melanoma *in vivo* may incorporate antibodies masking CD59, or determination of CD59 levels in patients to test whether a complement mediated strategy is of potential benefit to them.

Neutralisation of the CD59 complex is another strategy that has been explored. Biotinylated anti-CD59 monoclonal antibody was directed against the tumour cells with a high affinity biotin avidin bridge using a fragment of R24 as a targeting monoclonal antibody. The biotinylated anti-CD59 mAb lost its ability to activate complement but was able to neutralise CD59 activity. This prevented non-specific lysis of surrounding erythrocytes and endothelial cells whilst neutralising the CD59 activity of tumour cells and enabling complement mediated lysis (Junnikkala *et al.*, 1994). Therefore one venue to be explored to increase the efficacy of our therapeutic antibodies is the neutralisation or masking of CD59 to increase complement mediated lysis.

5.4.6.3 Antibody Conjugates Mimicking Components of Immune Pathway

An alternative approach to improve the initiation of the immune response is to use a conjugate that mimics a component of the immune pathway thus increasing the therapeutic efficacy. For example, Juhl *et al.* (1997) used cobra venom factor (CVF) as a conjugate to increase complement mediated lysis by anti-tumour mAbs. CVF is a structural and functional homologue to the third activated component of complement. Thus an antibody fused to CVF will mediate more effective complement lysis than the unmodified antibody. The authors showed that antibody-CVF conjugates mediated

selective complement dependent lysis of neuroblastoma cells. Furthermore, maximal cytotoxicity was observed at 7 hours, consistent with the kinetics of the alternative complement pathway. The maximal cytotoxicity observed was 95% with a single fusion molecule and 100% in synergy with the 3 fusion molecules tested.

Chapter 6

Discussion

6 DISCUSSION

The Noble prize awarded to Behring and Kitasato (1901) for their ground breaking work on passive immunotherapy sparked interest in a field that was given a new lease of life by Kohler and Milstein (also awarded a Noble prize in 1984 for hybridoma technology). The invention of PCR by Mullis and co-workers provided the final piece that has led to modern antibody technology now making up over 30% of biopharmaceuticals in clinical trials (Souriau *et al.*, 2003).

In the United States 95,880 new cases of melanoma have been diagnosed in 2004, of which 40,780 have been *in situ* (noninvasive) and 55,100 invasive (29,900 men and 25,200 women). Last year there has been a 4 percent increase in new cases of melanoma from 2003 (American Cancer Society's 2004 Facts & Figures). The seriousness of melanoma is demonstrated by the risk of recurrence. It is estimated that following surgery approximately 60% of patients with thick primary lesions (AJCC stage IIB) and 75% of patients with regional nodal metastases (AJCC stage III) suffer from recurrence of melanoma (Balch *et al.*, 2001). Median survival after the onset of distant metastases is 6 to 9 months, with less than 5% surviving 5-years (Balch *et al.*, 1992). Therefore, there is a imperative need for the development of an adjuvant therapy that may prevent recurrence of melanoma.

The treatment of malignant melanoma has undergone a revolution in the past few decades. Development of new technologies has meant that vaccines and genetically modified immunomodulatory agents are becoming available for the treatment of metastatic melanoma, once thought to be hopeless, provide a ray of hope for patients. However, radiotherapy, chemotherapy or immunotherapies alone have so far failed to provide a suitable therapy that can treat patients with advanced stages of melanoma (Campoli *et al.*, 2004). This is discussed in more detail in Chapter 1 Section 1.2.

6.1 SUMMARY

We have attempted to develop an adjuvant immunotherapy for melanoma. In the process we addressed three main areas. Firstly, we developed a scFv protein A fusion molecule that would mediate melanoma specific cytotoxicity via ADCC and CDC. Secondly, the unexpected finding of the immunogenic properties of scFvs led to intensive epitope mapping attempts mainly by Western blot detection. Finally, the therapeutic potential and side effects of both R3ZZ and R3 scFv were tested *in vivo*.

6.1.1 Protein A Fusion Molecule

Our research focused on developing an adjuvant immunotherapy for malignant melanoma. We used the RAFT3 scFv and fused it to 'ZZ', two artificial domains of *Staphylococcus aureus* Protein A. The R3ZZ fusion protein was unstable and difficult to express in *E. coli*. However, yields of 1mg/l pure protein were obtained with a good bacterial preparation. Several attempts were made to reduce the erratic growth of bacteria expressing R3ZZ. These included cloning different domains of protein A and testing expression in different vectors such as pBAD. However, these attempts were unfruitful.

PCR designed for the cloning strategies induced cross priming of highly homologous protein A domain gene segments. This meant that it was difficult to clone different domains of protein A. pBAD is a highly regulated vector for expression of toxic proteins in *E. coli* (Guzman *et al.*, 1995). However, cloning RAFT3ZZ into pBAD resulted in a much-reduced yield of RAFT3ZZ. Therefore, we concluded that toxicity may not be the main problem with RAFT3ZZ expression. My successor has constructed the pEZZ18 vector (used to clone the RAFT3ZZ) without the protein A promoter. Improved expression of scFv ZZ fusions was observed for some fusion molecules but not in others. Therefore, the erratic expression may be due to a combination of factors contributing to the properties of RAFT3ZZ and each aspect needs to be eliminated in a methodical manner.

The R3ZZ showed good binding to both human and mouse IgG and melanoma cells. We observed 65% CDC at 48 hours with 12.5µg/ml RAFT3ZZ and 85% ADCC at 4 hours with 0.5µg/ml human IgG and an effector to target ratio of 200:1. These

findings were comparable with results obtained by Holliger *et al.* (1997) using serum redirection by an anti-CEA x anti-complement diabody. Bergman and co-workers (2000) also observed similar *in vitro* ADCC in IL-2-activated NK cells with unmodified anti-ganglioside mouse IgG₃ monoclonal antibody (mAb) 3F8.

6.1.2 Immunogenicity of scFvs

The finding that scFvs are immunogenic brought an unexpected twist to our project and subsequently resulted in the development of assays to map the epitope. Mapping the epitope was no trivial matter and produced complex data that were difficult to interpret. We considered a number of different epitope mapping strategies and chose to locate the epitope by Western blot as a first step. Although this method has its limitations (e.g. being unsuitable for native epitopes) it allowed a number of potential epitope locations to be tested simultaneously on a panel of scFv derivatives. This was an important consideration as the available mouse serum was a limiting factor. At the end of this study we concluded that it is possible that there is more than one epitope located on the scFv. Based on these preliminary data we believe that there could be more than one B cell epitope that is located predominantly on the V_K, c-myc/his₆ tail and / or at the bottom of the scFv.

6.1.3 Therapeutic potential of R3ZZ and R3 scFvs

The therapeutic potential of the fusion molecule R3ZZ and the RAFT3 scFv were tested in balb/c nude mice. R3ZZ induced a significant growth reduction with a single dose of therapy, but upon repeated administration no significant improvement was observed. Similarly, the RAFT3 scFv plus 9E10 mAb had a therapeutic effect with a single dose resulting in a 65% tumour growth reduction. However, repeated administrations were not as effective as was hoped. Due to time constraints we were unable to optimise the dosing regimes for both R3 scFv and R3ZZ. This was mainly because the *in vivo* experiments were time consuming and there were some unforeseen administrative problems with the animal house unit.

In conclusion, our work investigated two possible adjuvant therapies for malignant melanoma. Both have shown to have a therapeutic effect in an *in vivo* model.

However, due to time constraints the extent of their therapeutic effect *in vivo* has yet to be explored more fully.

6.1.4 Fusion Molecule vs Immunogenic scFv Therapy

Having studied two potential therapies for melanoma by serum IgG retargeting it is important to address which of the two therapies may be a more suitable form of treatment. Both the R3ZZ and the immunogenic R3 scFv have potential advantages and disadvantages for use in therapy.

6.1.5 *In Vitro*

In vitro analysis showed that there is no significant difference between R3 and R3ZZ in terms of protein stability and production. R3 scFv offers an advantage due to the stable expression in *E. coli*, whereas the expression of R3ZZ was highly variable even after a number of attempts to make a more stable variant. However, when R3ZZ was produced it had an increased yield of protein and when purified was more stable than R3 scFv. The *in vivo* data showed a more significant tumour growth delay with R3ZZ therapy in comparison with R3 scFv.

Establishing suitable cytotoxicity assays proved to be difficult, laborious and time consuming. The CDC assay could be optimised further by using serum and protein ratios similar to those *in vivo*, as this would enable a more realistic extrapolation to a clinical setting. The ADCC assay could be optimised further by using fresh PBMCs to test cytotoxicity, as this again, would enable a more realistic extrapolation to a clinical setting. Both the results from ADCC and CDC could have been confirmed using different cytotoxicity assays. The ideal assay to use would be the chromium release assay as it is the most commonly used in the literature and background through the use of serum is not a significant problem with this particular assay.

6.1.6 Clinical Setting

The potential of therapies using RAFT3 and RAFT3ZZ when extrapolated to the clinical setting is quite different as they work by different mechanisms. R3ZZ is a therapeutic fusion molecule and therefore would start to act as a therapy immediately.

By contrast, the R3 scFv is an immunogenic protein not originally intended for therapy (even though it can function as a therapeutic molecule). A patient would need to be immunised with the scFv twice before an adequate anti-scFv antibody titre develops and the scFv could exert a therapeutic effect. If the two immunisations were administered one month apart then a patient would require 2 months before the scFv could be used for the actual therapy. This could prove critical for stage IV patients who only have an approximately 6 months survival expectancy. Furthermore, it is possible that the immune system of late stage patients is compromised e.g. following chemotherapy and this could limit the therapeutic potential of scFvs further. Therefore unmodified scFvs would seem a more realistic form of therapy for high-risk early stage patients who have no macroscopically detectable metastatic spread. By contrast, R3ZZ could be administered as a therapy for all disease stages.

Another important consideration is that early antigen recognition by T cells can lead to an elimination of aggressive melanoma cells with metastatic potential, since patients with lymphocyte infiltration have a better prognosis (Clemente *et al.*, 1996). Alternatively, it can be argued that there is a selection in favour of cells with greater malignant potential expressing antigens not recognised by infiltrating lymphocytes or having lost expression of target antigens. This has been correlated with partial regression of melanoma and some studies have shown that this could be a bad prognostic factor (Clark *et al.*, 1989).

Leonetti *et al.* (1998; 1999) have shown enhanced immunity and T cell activation by a snake toxin fused to ZZ. By contrast, we found a reduced immunogenicity of RAFT3 scFv after fusion to ZZ that could in part be due to a lower T_H response. Furthermore, the nude mice used in our therapeutic *in vivo* model are T cell deficient. Therefore, the role of T cells in R3ZZ therapy will require further studies.

6.1.7 Disadvantages of Immunogenic scFvs

The immunogenic properties of scFvs impose many limitations, particularly as most of the current uses of scFvs rely upon their presumed weakly immunogenic characteristics. For example, a large number of scFvs have been developed for radioimaging including the RAFT3 scFv (Kang *et al.*, 1999). However, due to their

immunogenicity in their present form scFvs cannot be used for repeated imaging and therefore would not allow the progress of the disease or success of therapy to be monitored. An immune response against scFv would alter the biodistribution characteristics of scFvs resulting in increased non-specific normal organ background.

6.1.8 Extrapolation of Mouse data to Humans

Although all mammals are very similar in their overall body plan, there are some differences in the details of both development and metabolism, and occasionally these differences can prevent the extrapolation of mouse data to humans and vice versa (Erickson, 1989). The *in vivo* data obtained in rodents cannot be relied upon 100% to be extrapolated to humans. This is discussed in detail in Chapter 5.

The *in vivo* models used in the present study were balb/c nude mice for the therapeutic studies and CBA mice for the immunogenicity studies. The *in vivo* models chosen for this study were based on both availability in the animal unit and because they are standard mouse models. However, it would also be possible to use SCID mice as a therapeutic model. SCID mice accept human lymphocyte xenografts and can be used as a tumour immunotherapy model with a semi-functional human immune system (Mule *et al.*, 1992). However, this is a technically demanding mouse model and was therefore unsuitable for our initial studies.

6.2 FUTURE DIRECTIONS

Although our data, both *in vitro* and *in vivo*, are promising due to time constraints a number of questions remained unanswered.

6.2.1 Making Non-immunogenic scFvs

Since they were originally developed for imaging it will be crucial to make scFvs non-immunogenic. One possible strategy is to make scFvs non-immunogenic by covering the epitope to create a diabody or tribody version, provided that the epitope was located at the bottom of the scFv as we suspect.

6.2.2 T_H Mutation

It is also possible to mutate and destroy T_H epitopes on scFvs by site directed mutagenesis using computer programs to predict the epitope locations (Schirle 2001). Another possibility is to align the V sequence of both mouse and human scFvs, locate key amino acids within the T_H epitopes and then exchange mouse amino acids by their human counterparts, thus making the scFvs non-immunogenic in humans (Mateo *et al.*, 2000). A disadvantage of this method is that success of deimmunisation cannot be tested in a mouse model. Biovation, the company that developed this approach claim that treatment of patients with a monoclonal antibody consisting of deimmunised mouse V regions and human constant regions does not trigger an immune response (www.biovation.co.uk). However, the results have not been published yet.

6.2.3 Combination Chemotherapy with Immunotherapy

The combination of dacarbazine with IFN- α appears more active than standard single-agent dacarbazine in metastatic melanoma (Huncharek *et al.*, 2001). Konjevic and co-workers (2003) investigated the NK cell activity, expression of cytokines (IL-2 and IFN- α) and activation of antigens during chemo-immunotherapy with dacarbazine and IFN- α . The authors observed that there was a significant increase in NK-cell activity, CD4⁺ T_H cells, CD4/CD8 T-cell ratio and expression of activation antigens CD69 and CD38 on NK and T cells, respectively. However, following subsequent cycles of therapy there was a significant increase in activation antigens without an increase in

NK cells. The limited effect observed was believed to be due to the adverse effect of high levels of TNF- α in melanoma. This study highlights the need to investigate the role of cytokines in melanoma kill by R3ZZ and R3 scFv and their potential for combination treatment.

The main disadvantage associated with combined immunotherapy is that of side effects. DeGast *et al.* (2003) demonstrated that combined immunotherapy with GM-CSF, IL-2 and IFN- α leads to flu-like symptoms with transient liver function disturbances. However, the toxicity observed was manageable on an outpatient basis. These findings were similar to Groenwegen *et al.* (2002) who used dacarbazine with GM-CSF, IL-2 and IFN- α as sequential chemo-immunotherapy. These results are promising as they provide evidence that use of immunotherapy with cytokines is a real possibility with manageable side effects.

6.2.4 Combination Antibody Therapy with Cytokines

A number of studies have shown that co-administration of antibodies with cytokines can improve the therapeutic efficacy of antibodies. Ansell, 2003 presented a review of concurrent administration of IL-12 on the efficacy of rituximab in B-cell non-hodgkin lymphoma. The review concluded that IL-12 appears to have significant clinical activity with a high clinical response rate in early phase clinical trials. Furthermore, studies have shown that combinations IL-2/IFN- α , IL-2/IFN- γ , IL-2/IL-12, and IL-12/IFN- α potentiated ADCC whereas, IL-4 reduced the IL-2, IL-12, and IFN- α induced ADCC (Flieger *et al.*, 1999; Flieger *et al.*, 2000).

6.2.5 Radioimmunotherapy

Radioimmunotherapy uses therapeutic radioisotopes to augment the cytotoxic activity of monoclonal antibodies. It is a significant advance in the effort to harness the specific cell targeting of monoclonal antibodies to deliver highly cytotoxic therapy targeted to malignant cells. This approach has been tested in clinical trials using antibodies labelled with different radioisotopes directed at the same or different cell surface antigens.

The increased response rates observed with radioimmunotherapy over unlabeled monoclonal antibodies are attributable to the ability of radioisotopes to mediate tumour cell lysis and eradicate adjacent malignant cells. This is referred to as the 'crossfire effect' (Hendrix *et al.*, 2002). Response rates in clinical trials conducted among patients refractory to other therapies were considerably higher than those achieved with an unconjugated monoclonal antibody (Davis *et al.*, 2000; Knox *et al.*, 1996; Wiseman *et al.*, 2000).

The efficacy of monoclonal antibodies labelled with radioactive isotopes has been tested in clinical trials for more than 10 years. Yttrium 90 (^{90}Y) ibritumomab tiuxetan (Zevalin) is the first of these products to be approved by the US Food and Drug Administration (FDA) for the administration of low-grade B-cell non-Hodgkins Lymphoma.

Another possible direction for the future could be to use scFvs for the radioimmunotherapy of melanoma by developing radiolabelled immunotoxins capable of targeting tumour cells by two different mechanisms (Manske *et al.*, 1988; Buchsbaum *et al.*, 1987; Ito *et al.*, 1991). The addition of a radionuclide to the fusion protein R3ZZ could enable the lysis of tumour cells that escaped ADCC or CDC by R3ZZ. There are a number of suitable α , β and γ -emitting radionuclides, including isotopes emitting β -particles such as ^{131}I , ^{90}Y , ^{186}Re , ^{188}Re and ^{57}Cu (Casey *et al.*, 1999; Syme *et al.*, 2003). Isotopes emitting α -particles (^{211}At , ^{212}Pb and ^{212}Bi) have been shown to release ten times greater energy than β or γ -emitters and are able to destroy tumour cells whilst leaving healthy cells in tact (Kennel *et al.*, 2002; Wesley *et al.*, 2004).

This approach has been developed further by pretargeting bispecific scFvs so that one binding site would bind to a melanoma antigen whereas the other binding site could then be targeted by radiolabelled molecules such as haptens. This pretargeting approach can be modified further by conjugating streptavidin or biotin to the preadministered scFv, followed by the injection of radiolabelled biotin or streptavidin (Paganelli *et al.*, 1993). This strategy has been developed further using biotinylated antibody administered first, followed by avidin and streptavidin and finally radiolabelled biotin (Paganelli *et al.*, 1998; Paganelli *et al.*, 1999).

6.2.6 Bispecific Antibodies

Kipriyanov *et al.* (2002) demonstrated a synergistic effect of small recombinant bispecific molecules recruiting different populations of human effector cells (NK and T cells) to the same tumour target. Synergy has been demonstrated in a number of studies using bispecific diabodies targeting two different antigens, e.g. CD19 x CD3 and CD19 x CD16 in SCID mice for the treatment of B cell lymphoma (Cochlovius *et al.*, 2000) and Non-Hodgkin's lymphoma (Kipriyanov *et al.*, 2002). Bispecific anti-CD19 x CD3 antibody with anti-CD28 mAb to act as a co-stimulatory molecule for T cell activation (Chambers *et al.*, 1999), as well as CD30 x CD3, CD30 x CD28 and CD30 x CD16 antibodies have also been shown to synergise in an *in vivo* model for Hodgkin's disease (Renner *et al.*, 1995; daCosta *et al.*, 2000). Synergising effector cells that can be recruited using this strategy include monocytes and macrophages (CD16), NK cells and T cells (CD3, CD28) (van Ojik *et al.*, 2001).

Therefore, one other area that could be explored further is to use serum IgG redirection in combination with bispecific diabodies that would recruit different effector cells. In particular, R3ZZ could be used together with diabodies that activate T cells as these are not recruited by R3ZZ mediated ADCC.

An example of this has been published by Holliger and co-workers (1997). The authors constructed a diabody with one arm directed against the tumour antigen CEA and the other against serum Ig. The bispecific diabody could recruit complement, induce mononuclear phagocyte respiratory burst and phagocytosis and promote synergistic cytotoxicity towards colon carcinoma cells in conjunction with an anti-CEA x CD3 diabody.

6.2.7 Mapping B Cell Epitopes

In our study we used mouse sera in attempts to map the B cell epitope(s) on scFv. ELISA and Western blot were used to locate the epitope since these techniques were already established in our lab. Furthermore, Western blot had the advantage that it allowed screening of a large number of clones with relatively small amounts of immune sera. However, Western blot also limited the analysis to non-native, linear epitopes. On the other hand, denaturation could uncover epitopes concealed in native protein. Finally, the data produced with this assay were complex and inconclusive.

In view of these results it may be more suitable to establish alternative ways of epitope mapping in order to determine the location(s) of the epitope(s) on scFv. One alternative approach would be the generation of anti-scFv hybridomas. A number of techniques have been described for mapping epitopes recognised by hybridomas (Williams *et al.*, 2000; Ito *et al.*, 2003). We also attempted to make hybridomas with splenocytes obtained from RAFT3 immunised mice but were not able to obtain any conclusive data. Due to time constraints this aspect of the project was not pursued further. However, the production of anti-scFv hybridomas will be essential for epitope fine mapping.

6.2.8 Mapping T cell Epitopes

A common approach for mapping T cell epitopes is to synthesise peptides with overlapping sequences and test them for their ability to stimulate T cell proliferation (Shreffler *et al.*, 2004; Muhle *et al.*, 2004). Other methods for T cell epitope mapping that may be investigated further include pepscan assay, site directed mutagenesis, prediction algorithms e.g. synpethi index, antigenicity index. This is discussed in more detail in Chapter 4 section 4.2.14.

6.2.9 Antigen Shedding

Both the R3 scFv and the R3ZZ therapy depend on the recognition of high molecular weight melanoma associated antigen (HMW-MAA). Therefore, it is import to ensure that antigen shedding is limited as much as possible. Factors known to increase surface expression, total content and shedding of HMW-MAA from melanoma cells

include IFN- α (Giacomini *et al.*, 1983; Giacomini *et al.*, 1984), TGF- β (Heredia *et al.*, 1996a; 1996b) and hyperthermia (Giacomini, *et al.*, 1983). IFN- γ has also been shown to induce a slight reduction in the expression of the HMW-MAA (Tsujiaki *et al.*, 1987).

Therefore, it is important that due consideration is given to the possibility of shedding of HMW-MAA when administering cytokines to enhance the therapeutic efficacy of the R3ZZ fusion molecule or the R3 scFv as a therapy.

6.3 CONCLUSION

In conclusion, this study has begun to evaluate two potential adjuvant immunotherapies for malignant melanoma.

This paves the way for a novel therapy using scFvs as therapeutic molecules that has not been demonstrated previously. Furthermore, the finding that scFvs are immunogenic and potentially therapeutic opens a realm of new possibilities for developing therapies against cancers where scFvs are available, but have not been tested in a therapeutic context. Therefore, this finding could have significant impact on the rapid progression of new and successful adjuvant therapies for a number of cancers.

We have also shown that fusion of scFv with *Staphylococcus aureus* protein A has a therapeutic effect with limited side effects. Both these therapies have shown a strong potential to be developed further, possibly in synergy with each other, to provide an adjuvant therapy that would be effective in preventing the formation of micrometastases following surgery and the recurrence of melanoma.

CHAPTER 7

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REFERENCES

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